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RETARDED GROWTH, LIFE SPAN, ULTIMATE BODY SIZE AND AGE CHANGES IN THE ALBINO RAT AFTER FEEDING DIETS RESTRICTED IN CALORIES [1]

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FOUR FIGURES

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The literature concerning retarded growth and length of life has been reviewed recently (McCay, '39). The present experiments represent an attempt to verify our earlier findings (McCay, Crowell and Maynard, '35) concerning the interrelationships between length of life, the retardation of growth and the effect of long retardation upon the ability to resume growth and attain a normal body size.

Diets used in these studies were made very high in percentage of such constituents as protein, minerals and vitamins in order to insure an adequate ingestion of these essentials. The retardation is effected by the deficiency in the daily allowance of energy in the diet. In the present study every animal was given the same daily allowance of the basal diet. Animals permitted to grow normally were given additional calories.

In none of our studies have we any evidence that the normal animals have their life spans shortened due to the richness of the diet because our values for these fall within the ranges found by Sherman and Campbell ('37), Slonaker ('35) and Drummond et al. ('38).

[1] These studies were started with support from the Snyder Grants made by Mrs. Harry Snyder and were continued under the Rockefeller Grant for Research in Longevity.

The amount of this diet fed to individuals daily was determined by the amount needed to maintain the retarded animals at a stationary body weight. The results of such a regime insure the ingestion of approximately equal amounts of such essentials as protein and minerals, but do not impose any additional burden upon such organs as the kidneys in the case of animals allowed to grow normally. In this case all animals ingested enough protein, vitamins and minerals for growth, but the retarded animals could not grow due to a deficiency of calories.

The composition of the basal diet was the following: cooked starch, 22; cellulose, 2; lard, 10; sucrose, 10; salt mixture (O. and M.), 6; cottonseed oil, 5; yeast, 5; casein, 40.

For vitamin supplements in the present case the control rats that were destined to grow normally were divided into two equal groups. Each member of the first control group was fed a 1% solution of carotene in cottonseed oil in an amount to allow 15 'A' units (A.D.M.A.) per rat per day. In addition, an allowance of $\frac{1}{3}$ gm. of irradiated yeast was given each individual three times weekly. This same allowance of supplements was given each retarded rat. The second control group was fed 3 drops of cod liver oil daily with $\frac{1}{3}$ gm. of dried yeast (not irradiated) fed three times weekly.

The animals allowed to mature at any time were fed additional calories in the form of a mixture of sucrose, cooked starch and lard in the following proportions: 38:57:5.

The plan of the experiment consisted in dividing 106 rats at the time of weaning into two groups, one containing thirty-three members, to serve as a normal control group, and one of seventy-three to be retarded for varying periods. The normal group was then subdivided into two, containing seventeen and sixteen members. The purpose of this subdivision was to have one fed cod liver oil throughout normal life, and the other fed irradiated yeast and carotene for fat-soluble vitamin supplements.

The retarded animals were not subdivided further until the first period of 300 days had passed. At the end of 300

days there were only thirty-eight of the original seventy-three retarded rats alive as well as all of the original thirty-three controls that had been allowed to grow normally.

These losses in the retarded group were due to two failures in the heating system. Under such conditions the normal animals that were well fed could withstand the drastic drop in temperature of the room while the retarded ones that were being kept at maintenance level had little reserve and part of them perished.

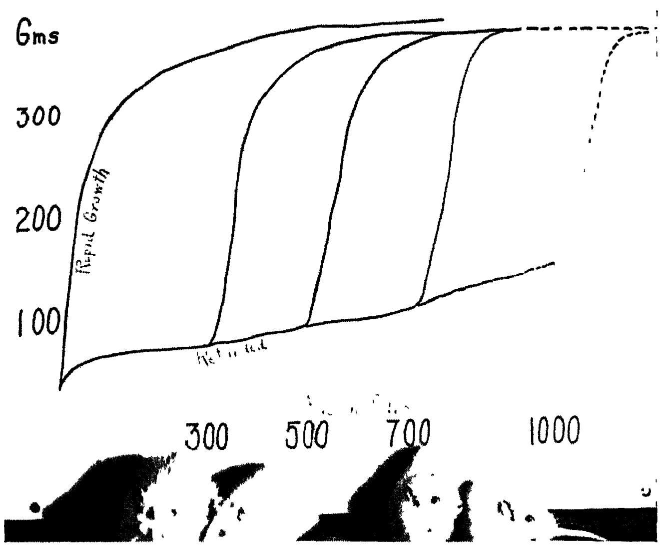

Fig. 1 Projected growth curves showing the plan for the experiment and representatives of the retarded groups at the age of 1000 days.

At the end of 300 days the retarded animals were divided into four groups as nearly equal as possible. One was fed additional calories to permit growth. The other groups were thus assigned at this date to the several periods for resuming growth. These were at the end of 500, 700 and 1000 days. The design of the experiment is shown in figure 1. These curves were drawn in advance and used as a background to photograph typical representatives of each group. In the copy shown in figure 1, the experiment had been in progress

for 1000 days, and all members of the normal group had died while representatives of each of the others were still alive.

The general plan for feeding the retarded animals was to allow them to increase in body weight to the extent of 10 gm. at intervals of about 100 days. The period of maintenance at a fixed body weight is given in table 1. The increase in weight was allowed to take place during a period of a week or 10 days. Fresh liver was fed during this period to allow calories for this growth. Equal amounts of liver were given the controls.

TABLE 1

Period of life of retarded rats during which they were maintained at given levels of body weight

BODY WEIGHT LEVEL *gm.*	AGE OF MAINTENANCE *days*
40	25– 44
60	54– 117
70	130– 228
80	235– 329
90	339– 443
100	450– 548
110	555– 688
120	696– 716
130	731– 822
140	836– 892
150	904– 955
160	963–1004

In assembling data upon such values as ultimate body size for these groups certain individuals had to be excluded, such as members of the group retarded for 1000 days that died before attaining this age. These individuals can be included, however, in considering such factors as disease that caused death or the degree of calcification of the arteries and organs.

The data concerning body weight are in table 2. In general retarded animals were never able to attain the body weights of those that grew normally. This indicates that even in retardation for a period of 300 days there is a permanent stunting effect upon the rat body. In general, the females tend to be lighter in weight than the males even after they

have been kept at the same level during long periods of retardation.

The length of life for each individual is given in table 3. In the groups that grew to maturity normally the death rate of the males is about that anticipated from such a limited number. The females in these groups died prematurely, however. From earlier experience about 20% of them should have exceeded 1000 days, but all of them were dead before this time.

TABLE 2

Maximum weights in grams by groups and subgroups

GROUP	SEX	MEAN BODY WEIGHT AT TIME OF REALIMINATION	MEAN MAXIMUM WEIGHT ATTAINED	MEAN AGE OF ATTAINING MAXIMUM WEIGHT	MAXIMUM WEIGHT ATTAINED BY ANY INDIVIDUAL	NUMBER OF ANIMALS CONSIDERED
				days		
Normal growth	♂		450±15	586±24	555	9
Fed carotene	♀		321±16	446±32	416	8
Normal growth	♂		445±17	543±47	567	8
Fed cod liver oil	♀		372±24	572±45	536	8
All retarded	♂		327±18	832±25	485	15
	♀		260±10	846±42	365	16
Retarded 300 days	♂	79	415±15	709±38	443	4
	♀	79	244±11	603±70	300	5
Retarded 500 days	♂	105	371±36	801±32	485	4
	♀	104	298±16	849±20	350	5
Retarded 700 days	♂	148	287±27	844±36	390	4
	♀	138	247±30	985±64	365	4
Retarded 1000 days	♂	179	209±22	1023±20	270	3
	♀	183	232±21	164±37	263	2

Formula used: $PE_m = 0.6745 \sqrt{\frac{\Sigma fd^2}{n(n-1)}}$ $(f = 1)$.

The mortality curve for those fed cod liver oil is more nearly normal than for those fed carotene and irradiated yeast as sources of vitamins A and D. None of these data indicate any unfavorable effect of cod liver oil when fed during the entire life span.

The retarded groups all had members alive when the last of the normal controls had perished. These retarded groups

also contained members that far exceeded the normal life expectancy.

The period of adult life tends to be much shorter after long retardation. A brief table (4) will show this. The period of life remaining after maturing becomes progressively less as the length of the retarded period increases.

TABLE 3

Life span of individuals in days (those living over 300 days). The figures below the line represent those rats still alive when the last control died

NORMAL GROWTH FED CAROTENE		NORMAL GROWTH FED C L.O.		RETARDED 300 DAYS		RETARDED 500 DAYS		RETARDED 700 DAYS		RETARDED 1000 DAYS	
♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
377	475	308	404	805	555	366	793	772	406	336	815
643	527	439	624	815	598	721	1008	782	697	859	846
673	536	506	636	822	711	856	1028	915	867	1004	1152
703	555	627	646	1018	1007	986	1043	1025	1045	1086	1320
713	570	668	722		1183	1103	1078		1134	1127	
720	635	809	808						1320		
767	650	869	844								
784	682	886	965								
896											

TABLE 4

Length of life after attaining maturity

RETARDED IN DAYS	MEAN AGE ATTAINED BY THOSE ALIVE AT 300 DAYS	TIME LIVED AFTER COMPLETING GROWTH *days*
300	835	535
500	895	457
700	896	253
1000	949	138

As a whole the effect of retardation of growth is similar to that obtained previously. A much higher per cent of the rats can attain ages that exceed 1000 days if they have been subjected to retardation. Thus from the retarded groups six males exceeded an age of 1000 days. These came from an original group of only thirty-eight males. No data are available to permit an estimate of the number of males needed in a normal population to secure this number at this age.

The line was drawn in table 3 to indicate the number alive in the retarded groups at the time of death of the last of the normal-growth animals. Retardation permits a higher per cent of animals to attain old age if the diet is qualitatively complete in other respects. This does not conflict with the data of Sherman and Campbell ('37).

These results in combination with those of the earlier report (McCay, Crowell and Maynard, '35) indicate that the method of retardation affords a useful technic for studying aging. Animals that are relatively very old become available for study. Furthermore, the parts of the body that age in a

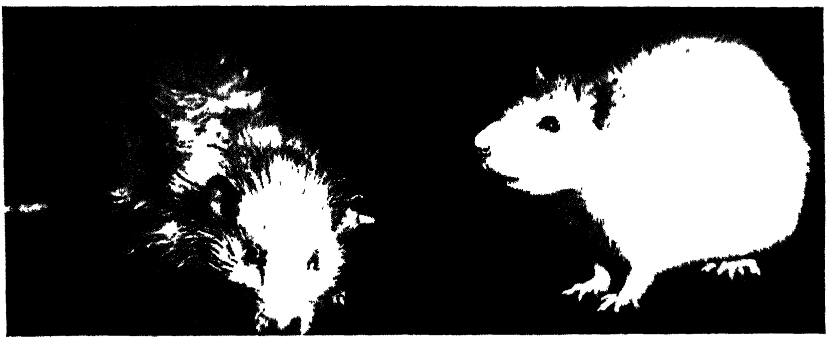

Fig. 2 Age 964 days. This photograph was taken the last day of life for the last of the controls on the left and 36 days before the retarded rat on the right was allowed to mature. Note the contrast in degree of senescence.

normal animal can be compared with those of retarded animals that are destined for a much longer span. This affords a tool for investigating the systems that break down in the normal process of aging.

The condition of the hair of the retarded animals tended to remain fine in contrast to the coarseness in the case of those that grew normally. This is evident in the photograph in figure 2. The retarded animal on the right in the picture was allowed to mature at 1000 days of age and lived nearly a year after this photograph was made.

The weights of the organs in table 5 indicate that the ultimate body size attainable decreases in proportion to the

TABLE 5

Effect of long retardation followed by realimentation upon the weight of organs

	NORMAL GROWTH FED CAROTENE		NORMAL GROWTH FED C.L.O.		ALL RETARDED		RETARDED 300 DAYS		RETARDED 500 DAYS		RETARDED 700 DAYS		RETARDED 1000 DAYS	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Mean body weight in grams less organs and gastro-int. tract	235	184	248	173	196	128	220	110	214	139	177	133	123	140
Mean weight of liver in grams	10.6	9.2	11.4	11.6	10.7	8.2	10.4	8.5	12.6	8.8	10.9	7.2	8.1	8.2
Mean weight of kidneys in grams	2.6	2.4	3.0	2.5	2.2	1.8	2.4	1.8	2.5	2.0	2.3	1.8	1.6	1.5
Mean weight of spleen in grams	1.4	1.3	1.0	1.2	0.9	1.0	1.2	1.0	0.8	1.0	0.9	0.9	0.6	0.7
Mean weight of heart in grams	1.6	1.4	1.3	1.4	1.3	1.2	1.4	1.4	1.4	1.1	1.2	1.0	1.1	1.6
Mean body length nose to anus (cm.)	21.4	20.1	22.3	18.5	20.2	18.6	21.2	18.6	20.6	19.5	20.0	17.7	18.3	18.0
Number of animals	7	9	8	8	15	16	4	5	4	5	4	4	3	2

period of retardation. There is probably no significant difference between the two normal groups. In general our findings accord with those of Jackson ('37) and co-workers in showing that retardation leads to a permanent decrease in the size of the body and its parts.

Bone growth

After careful dissection bones were allowed to air dry and used for determining the size and density. In the normal groups the ranges in density were the following: male humerus, 1.108 to 1.362; femur, 0.937 to 1.286; female humerus, 1.200 to 1.407; femur, 1.062 to 1.275. The bones of the males tended to become less dense with age while there was no change in those of the females up to 965 days.

A condensed summary of bone data for all groups is presented in table 6. As far as bones can serve as criteria these data indicate that the loss of the ability to attain a maximum body size is progressive with the period of retardation.

The permanent decrease in the body size after such a long period of retardation as 1000 days, even among the animals that could resume growth, was between 10 and 20% below the normal as far as the bone data indicate. This is illustrated in figure 3. Body length data also indicate that the better of the group retarded for this long period attained a length of about 85%. From the organ data, such as the weight of the organs or of the 'empty' body, the better animals seem to have attained a size of about two-thirds the normal. Such data are subject to considerable variation, however, because the animals were both old and diseased at the time of death.

The density of the bones from retarded animals is low. This is partly due to the great age at which these animals die. The mean density for both sexes of the retarded animals was 1.21 for the humeri. In the case of retarded animals there is considerable variability and in animals over 1000 days of age the density of the humerus varied from less than 1 to more than 1.3. No explanation can be offered for the absence of sex differences in the retarded groups except that

TABLE 6

Changes in the size and weight of bones after retardation followed by growth

DIET	SEX	BONES	LENGTH	WEIGHT (AIR DRY)	WEIGHT (OVEN DRY)	DENSITY (AIR DRIED)
			cm.	*mg*	*mg.*	
Normal growth 'carotene'	♂	Humerus	3.0	341	321	1.26
	♀	Humerus	2.7	235	268	1.35
	♂	Femur	3.9	702	666	1.10
	♀	Femur	3.5	586	552	1.22
Normal growth 'cod liver oil'	♂	Humerus	2.9	335	316	1.26
	♀	Humerus	2.7	263	250	1.33
	♂	Femur	3.8	691	644	1.10
	♀	Femur	3.5	540	509	1.20
Retarded 300 days	♂	Humerus	2.8	309	289	1.26
	♀	Humerus	2.5	238	222	1.21
	♂	Femur	3.7	633	597	1.18
	♀	Femur	3.3	453	428	1.13
Retarded 500 days	♂	Humerus	2.6	264	248	1.26
	♀	Humerus	2.6	237	222	1.20
	♂	Femur	3.5	535	502	1.18
	♀	Femur	3.4	471	442	1.13
Retarded 700 days	♂	Humerus	2.6	257	238	1.18
	♀	Humerus	2.5	202	189	1.24
	♂	Femur	3.6	553	522	1.15
	♀	Femur	3.1	391	368	1.11
Retarded 1000 days	♂	Humerus	2.5	219	212	1.15
	♀	Humerus	2.4	201	188	1.18
	♂	Femur	3.2	384	406	1.06
	♀	Femur	3.1	373	348	1.03

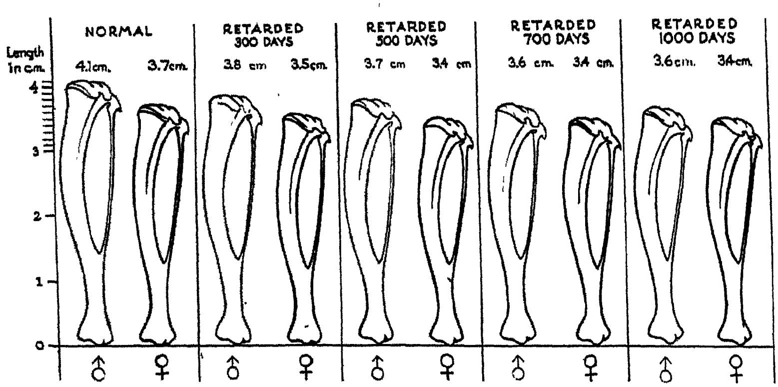

Fig. 3 The effect of retardation and realimentation upon the ultimate size of bones in rats of opposite sex.

they were kept at the same body weights. In both the previous report and this one the life span of the opposite sexes seemed to be the same in the case of retarded animals.

Bone growth after long retardation

The growth of the bones in rats that had been retarded for 700 days and more was followed by making x-ray photographs at frequent intervals. From these the length of the

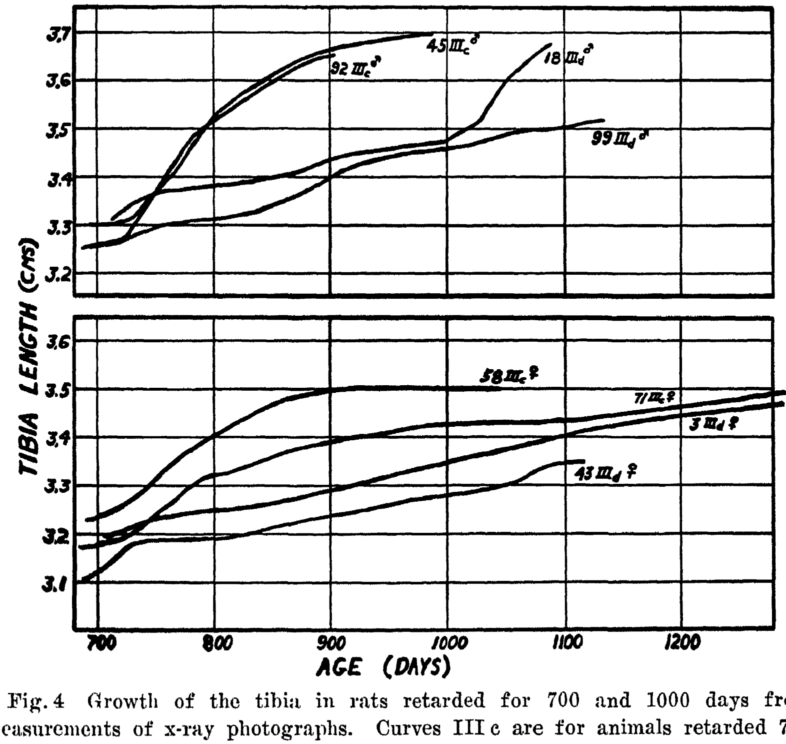

Fig. 4 Growth of the tibia in rats retarded for 700 and 1000 days from measurements of x-ray photographs. Curves III c are for animals retarded 700 days. Those marked III d were allowed to resume growth after 1000 days.

bones such as the tibia could be measured. Typical data from these measurements are plotted in figure 4. The upper curves for each sex show the rates at which the bones responded when the rats were allowed to grow after 700 days of age. After 700 days all retarded animals responded promptly when allowed enough calories for growth. Although the opposite

sexes were approximately the same in body weight at the beginning of this period the bones of the females were smaller. The bones of the males respond to the realimentation more promptly than those of the female. The bones of the male retain the power to grow larger than those of the female.

Of the rats retarded for 1000 days only three males and two females were alive at the date set for realimentation. Curves for four of these animals are marked III-d in figure 4. The first characteristic of these is the slow, regular increase in the length of the bones that was taking place even when the individual was held at a constant body weight. Even at these extreme ages the bone retains its power to forge ahead in growth while the other parts of the body remain stationary or shrink.

After these rats were fed adequately, part seemed able to accelerate the growth of the bone while others seemed to have lost this power. This is the first evidence that there is a time limit for retardation beyond which the body can no longer resume growth. At 1000 days some animals seem to retain this power of growth and others seem to have lost it. This is not the case in the earlier study when retardation exceeded a period of 900 days. Even after this extreme retardation of 1000 days in the present case the male is still able to grow to a larger body size than the female, as far as the skeleton indicates.

MAINTENANCE REQUIREMENTS FOR RETARDED GROWTH RATS

Jackson ('37 a) has discussed the requirements for maintenance of rats held at a constant body weight, recently. The results of the present study confirmed those of the earlier one inasmuch as the female at most levels needed more calories for maintenance than the male.

SUMMARY

Rats were retarded in growth for periods of 300, 500, 700 and 1000 days before being allowed to grow to maturity. Members of each of these groups were alive when the last of

the control groups had died at an age of 965 days. Retardation of growth by diets, complete except for calories, affords a means of producing very old animals for studying aging. Animals that are retarded for even 300 days can never become as large as those that mature normally. After 1000 days of retardation only part of the rats were able to resume growth when adequate energy was allowed in the diet. Even at this extreme period the male tended to grow to a larger size than the female. The growth of the bones in rats retarded for 700 and 1000 days was followed by means of x-ray photographs. The maintenance of a constant body weight in this period of old age does not check the growth of the bones. These increase slowly and respond to realimentation in all cases after 700 days of retardation but in only part of the cases after 1000 days.

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CHEMICAL AND PATHOLOGICAL CHANGES IN AGING AND AFTER RETARDED GROWTH [1]

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FOUR FIGURES

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In the preceding paper (McCay, Maynard et al., '39) a summary was given of the results of retarding rats for periods varying from 300 to 1000 days before permitting them to grow to maturity. In this report are incorporated the data concerning the changes in the chemical composition of some of the organs and a brief consideration of some of the pathology involved in these same animals.

CHANGES IN THE CALCIUM CONTENT OF THE EYES

The method used for calcium was that described by Ellis ('39). The tissues to be analyzed, chiefly eyes, were first dried to a constant weight at 105°C. All values were expressed in terms of per cent of the dried weight. Up to about an age of 1000 days the calcium of the eye varies between 0.019 and 0.092%. After 1000 days of age these values range between 0.079 and 1.67%.

In table 1 these data are assembled according to the age of the rat at death without regard to its previous treatment. The great increase in the calcium of the eyes in old age is more marked than that found in any other species. This literature has been summarized previously (McCay, '39).

[1] These studies were started with support from the Snyder Grants made by Mrs. Harry Snyder and were continued under the Rockefeller Grant for Research in Longevity.

The data for the calcium of the eye were studied from many angles. Retarded rats tended to live longer and the level of calcium was higher at the time of death. The eye calcium seems to increase in proportion to the time a rat has lived without regard to the retardation of growth. No correlation could be found between the level of calcium in the eye and the degree of calcification of such organs as the kidneys. There was no relation to the density of the bones.

In two cases where it was possible to dissect the lens, the calcium was lower than in the rest of the eye. Calcium was determined in the aortas from a few very old animals. On a dry basis these varied from 0.27 to 6.0%. No relation was found to the eye calcium.

TABLE 1

Age and calcium content of the entire eye

AGE GROUP	NUMBER OF RATS	MEAN PER CENT Ca
days		
506– 555	5	0.034
627– 697	8	0.034
703– 720	3	0.058
805– 886	9	0.043
965–1078	10	0.238
1103–1183	5	0.499
1320	2	1.078

THE TEETH OF RATS IN THE COURSE OF AGING AND AFTER RETARDATION

The condition of the teeth in rats is important in life span studies because they may serve as foci of infection or they may modify the consumption of food due to pain.

The jaws and teeth were examined under low magnification by a practising dentist. X-ray photographs of the jaws and teeth were also made.

The females seemed to suffer more consistently from the breakdown of teeth than the males. This was especially true in the normal animals. In both of these groups the mean age of the males exceeded that of the females. As a whole the

retarded rats showed increased destruction of teeth, but considering their advanced age they were remarkably free.

In the first control group fed carotene and irradiated yeast as sources of fat soluble vitamins, one male animal at the age of 896 days had lost two lower molars. Another female had a left molar that was carious to the gum line with the roots retained. In another female, dead at an age of 527 days, the the upper molars were normal while all but one of the lower molars had been completely destroyed. Similar conditions were noted in the second control group. One of the males in

TABLE 2

The effect of aging and retardation upon the condition of the teeth at death

GROUP	NUMBER AND SEX	MEAN AGE	NUMBER OF RATS WITH INTACT TEETH	PER CENT OF TEETH MISSING OR CARIOUS IN THE GROUP
Control	6 females	597	2	26
'carotene'	8 males	679	2	13
Control	8 females	600	0	33
'C. L. O.'	8 males	639	2	25
Retarded	4 females	717	1	23
300 days	4 males	865	1	23
Retarded	4 females	985	0	35
500 days	4 males	915	0	17
Retarded	4 females	935	1	23
700 days	4 males	873	0	25
Retarded 1000 days	3 females	937	0	36
or less	4 males	1019	1 (almost)	29

this group which had died at an age of 886 days had all teeth badly abraded. In one member of the group retarded for 300 days and dead at an age of 1007 days, all of the lower molars were gone, while the upper molars were normal except for loss of the alveolar process. However, in one case of those retarded for 1000 days, a male had nearly an intact set of teeth.

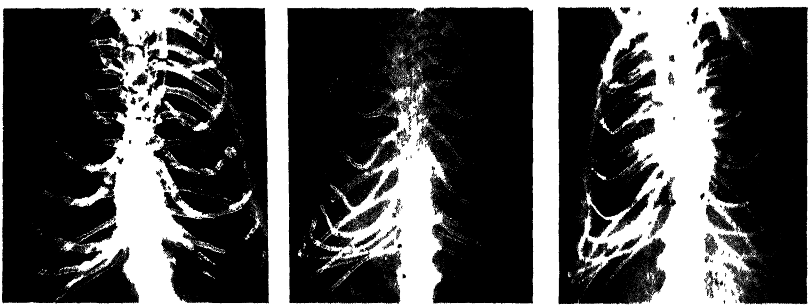
These data are summarized in table 2.

The variability among rats seems fully as great as that found in human beings.

THE CALCIFICATION OF COSTAL CARTILAGES

X-ray pictures were used to determine the degree of calcification of the costal cartilages. In animals dying at similar ages, calcification was much more marked in the control than in the retarded animals. Retardation of growth delays this calcification.

In figure 1 a is shown the oldest control, age 965 days, in figure 1 c a rat retarded for 1000 days, age 1086 days, and in figure 1 b another that was retarded for 1000 days but lived to an age of 1320 days. The greatest difference here is



a	b		c	
Control,	Retarded	1000 days	Retarded	1000 days
Age 965 days	Age	1320 days	Age	1086 days

Fig. 1 Heavily calcified costal cartilages of 'normal growth' rat on the left (a) contrasted with 'retarded' ones on the right (b and c).

seen between the control and the 1086-day-old rat. The 1320-day-old rat shows more calcification of the costal cartilages than the 1086-day-old rat, but not as much as the control.

If calcification is an index of physiological aging of cartilage, it then appears that the retardation affects this process.

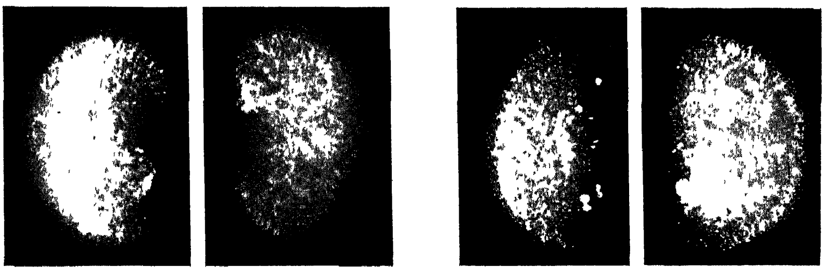
THE DEGREE OF CALCIFICATION OF THE HEART, AORTA AND KIDNEYS OF NORMAL AND RETARDED RATS

Observations were only made on those rats that lived more than 700 days. X-ray pictures were made, at the time of autopsy, of ten control and thirty-two retarded rats. The pictures were made with a tungsten target tube at a peak

voltage of 35 to 40 K.V. No filter other than the glass wall of the tube was used. The film was covered with two thicknesses of black paper. The calcification of the tissue of the kidneys, hearts and aortas will be discussed.

Kidneys

The x-ray stereograms of the kidneys make possible an estimation of the relative extents of calcification in the different parts of the kidney. The calcification, as illustrated in figures 2 a and 2 b, frequently extends through the pelvis, pyramid and medulla, but its appearance in the cortex is infrequent and never extensive.



a		b	
Retarded	500 days	Retarded	700 days
Age	1008 days	Age	1025 days

Fig 2 Extensive calcification in the kidneys of two retarded rats.

Table 3 was prepared from a study of the stereograms of the kidneys. It is not possible in most cases to distinguish calcium deposits in the pyramid from those in the pelvis so these regions of the kidney are considered as one. The extent of the calcification in each of the three regions was estimated on a basis of 0 to 3, 0 indicating no calcification and 3 indicating the most extensive case.

All of the control rats on which these observations were made died in the age range from 713 to 965 days. Twelve of the retarded rats died in the age range, 711 to 915 days. An examination of table 3 will show that both the incidence and

extent of calcification of the kidneys of the retarded rats are much higher than that of the controls of approximately the same age.

Eighteen of the retardeds died in the age range of 986 to 1320 days. Their kidneys were also extensively calcified but no more extensively than those of the younger group of retardeds.

TABLE 3

Incidence and extent of calcification in kidneys

NUMBER OF RATS IN GROUP	AGE AT DEATH	CALCIFICATION: Pelvis and pyramid Incidence	Pelvis and pyramid Extent	Medulla Incidence	Medulla Extent	Cortex Incidence	Cortex Extent
	days	%		%		%	
8 control	713 to 965	0	0	31	0.3	0	0
12 retarded	711 to 915	96	1.6	87	1.6	21	0.2
18 retarded	986 to 1320	95	1.7	86	1.7	17	0.2

Aortas

For making the x-ray pictures of the aortas, they were split longitudinally and laid flat on a piece of paper. Many of the aortas of the retarded rats showed extensive calcification. Examples of some of the more extensively calcified aortas are shown in figure 3.

Table 4 shows the incidence and extent of the calcification of the aortas of thirty-two retarded rats and ten controls. The incidence and averge extent of calcification are both markedly higher in the case of the retardeds, and as in the case of the kidneys the last eighteen retardeds that died are not appreciably different than the fourteen retardeds preceeding them. However, it should not be concluded from this that the calcification of the aortas parallels the calcification of the kidneys. Table 5 shows the incidence and extent of calcification of the aortas in relation to the time of retardation. The lowest incidence and the least extensive calcification of aortas is in the case of the controls, which were not

retarded at all. The highest incidence and the most extensive calcification is in the case of those rats retarded 1000 days. It should also be noted that there is a progressive increase in the incidence and extent of the calcification with increasing retardation. A similar comparison of calcificaton of kidneys

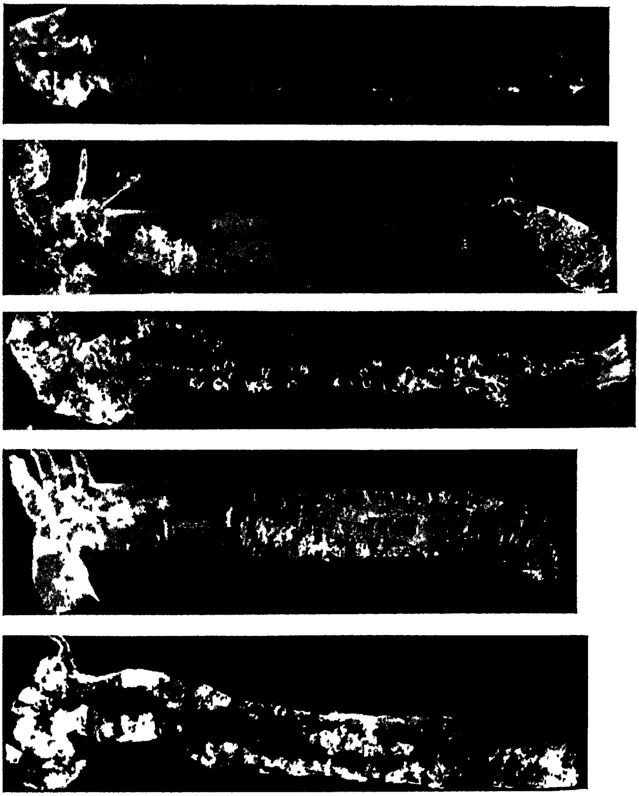


Fig. 3 Aortas of retarded rats showing calcification in the region of the arch. The lower pictures show calcification over the entire length.

shows no such effect within the retarded groups. The kidneys of the rats retarded for 300 days shows just as extensive calcification as those retarded for 1000 days. A preliminary report of the calcification of the aortas of rats was made by Hummel and Barnes ('38).

Hearts

X-rayed stereograms of the hearts of all of these rats were also made. Unfortunately, the auricles were removed from some of the hearts before the stereograms were made. Since some calcification seems to occur near the base of the heart, we cannot make as reliable comparisons as were made for kidneys and aortas. It may be said however, that the

TABLE 4

Calcification in aortas

NUMBER OF RATS IN GROUP	INCIDENCE OF CALCIFICATION OF AORTA	AVERAGE EXTENT OF CALCIFICATION OF AORTAS	RANGE OF AGES AT DEATH
	%		*days*
10 controls	20	0.2	713 to 965
14 retardeds	57	1.1	711 to 915
18 retardeds	61	1.2	986 to 1320

TABLE 5

Length of retardation period and calcification of the aorta

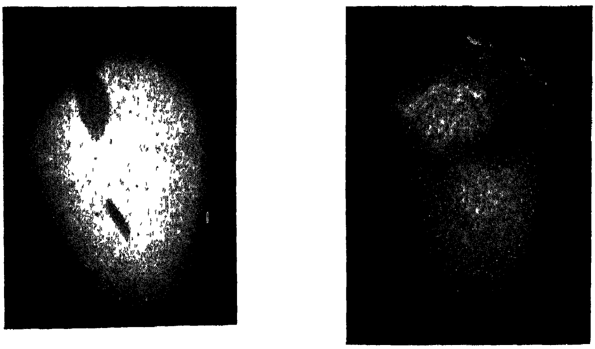
NUMBER OF RATS IN GROUP	LENGTH OF RETARDATION PERIOD	INCIDENCE OF CALCIFICATION OF AORTA	AVERAGE EXTENT OF CALCIFICATION OF AORTA	RANGE OF AGES AT DEATH
	days	%	%	*days*
10 controls	0	20	0.2	713 to 965
7 retardeds	300	29	0.4	711 to 1183
9 retardeds	500	44	1.0	721 to 1103
8 retardeds	700	62	1.4	772 to 1320
8 retardeds	1000 (or less)	100	1.9	815 to 1320

extent of the calcification in the hearts of the rats examined is much less, on the average, than that shown in the kidneys and aortas. A small calcification in the form of a ring near the base of the heart is frequently seen in both controls and retardeds. An example of this may be seen in the upper part of the heart shown in figure 4 a. This is the heart of one of the controls. If we exclude this ring we may say that the calcification in the hearts is more extensive and more frequent in the hearts of the retardeds. Figure 4 b shows the

heart of a retarded rat of the 1000-day group and a typical calcification in the auricles. This rat died at the age of 1004 days. Our x-ray data on the hearts are not sufficiently good to make comparisons of the kind we have made in the cases of aortas and kidneys.

GENERAL PATHOLOGY OF NORMAL AND RETARDED RATS

Limited observations indicate that our stock colony of rats suffers from a high incidence of lung disease when about 1 year of age. There is no way of detecting this ordinarily,



a b

Fig. 4 Slight calcification near the base of the hearts of control rat (a) and retarded rat (b).

however, except by dissection of the animals since they appear in fine health. From the few observations published elsewhere and from our dissection of rats from other sources, it seems that this is general. Nevertheless, it is a matter for serious consideration since it may be a complicating factor of marked importance when the life span is being studied.

If lung disease is important in terminating the life of rats considered normal, its influence must be much less in retarded animals. In the present study a high incidence has been found in both normal and retarded rats at the time of death. As a whole the condition of the lungs in retarded rats at extreme ages has been no different than that found in normal animals

that lived for a much shorter period. In rats retarded for 15 weeks Jackson ('37) found a lower incidence of both middle ear disease and lung disorder among retarded animals. The relation of rickets to rapid growth has long been recognized but the possibility of a similar relationship between diseases of bacterial origin has seemed remote. The growth rate may have a much more profound influence, however, than has been conceived.

The development of tumors in the course of the present study has been given some attention. In the groups permitted to grow normally seven out of fourteen females and two of the seventeen males died with tumors. Some of these tumors weighed nearly as much as the remainder of the animal.

Among the retarded animals only two tumors developed in the course of retardation. Both of these cases were among those retarded for more than 300 days. After retarded animals were allowed to mature, only five tumors were observed in twenty-five cases. Three of these five were in the group of nine rats allowed to mature at 300 days. This evidence is very limited but may indicate that retardation also decreases the number of tumors that develop. It is difficult to know whether the failure of these diseases to develop permits the retarded animals to live longer or whether the retardation modifies the composition of the body so that it is less subject to diseases.

The condition of the kidneys at the time of death varied widely among individuals in respects other than calcification. About a fourth of the control groups and those retarded for the extreme periods of 700 and 1000 days exhibited marked injury. This incidence of diseased kidneys seemed somewhat higher among those retarded for only 300 and 500 days.

SUMMARY

Rats that had been retarded for periods of 300 to 1000 days were used for studying the chemical and pathological changes that accompany aging. The calcium of the eyes was found

to vary from 34 to 1078 mg.% on a dry basis. Most of this calcium is deposited in the eyes after an age of about 900 days. The calcium of the eyes is not correlated with either changes in the bones or with the calcification of other tissues. The teeth of the rats were studied by means of the x-ray. Even at very advanced ages a few animals had intact teeth. About a fourth of all the teeth examined were carious or missing. Females exhibited more damage to their teeth than males although these females never reproduced. A number of cases with bad infections extending into the jaw bones were found. The calcification of the costal cartilages was followed by means of the x-ray. This calcification was much more marked in normal animals than in those of the same age that had been retarded in growth. X-ray photographs of calcified kidneys, hearts and aortas are included. The calcification of the aorta is found to have a much higher incidence among retarded animals than among normals. Indirect evidence indicates that retarded animals are less susceptible to lung disease when a year of age than the normals. Limited evidence also indicates a lower incidence of tumors in retarded animals.

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BONE DEVELOPMENT IN NORMAL AND RACHITIC RATS [1]

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THREE FIGURES

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The extensive use of the white rat for the assay of vitamin D warrants attention to the progressive changes in bone structure that take place during the development of the rachitic condition and during normal growth. Therefore, the object of the present investigation was to obtain a picture of the development of the radii of rats from 3 to 54 days of age on a stock colony ration, and of rats taken from the stock colony group and fed a rachitogenic ration from 24 to 54 days of age. Of the latter period, the portion from 24 to 47 days is that ordinarily used in this laboratory for the depletion of rats for use in vitamin D assays (U. S. P. XI, 1937, Rev.). The stock colony group will be referred to as the normal group.

EXPERIMENTAL PROCEDURE

The stock colony ration was one which has been in use in our colony for 10 years and consists of 60% ground whole wheat, 30% dried whole milk and 10% meat scrap (55% protein), with sodium chloride added to the extent of 2% of the weight of the wheat (Russell, '32). The rachitogenic ration was the well-known Steenbock 2965 (Steenbock and Black, '25).

[1] Journal series paper of the New Jersey Agricultural Experiment Station, department of agricultural biochemistry. Presented before the American Society of Biological Chemists at Memphis, Tennessee, April, 1937.

Radii were collected from two series of animals. The first series covered the period from January to June, 1936, and the number of animals in a litter varied from six to ten. The animals of a litter, nursed by mothers on the stock colony ration, were killed at 3-day intervals from 3 to 24 days of age, inclusive. Whole litters, weaned at 24 days of age, were continued on the stock colony ration or placed on the rachitogenic ration. The members of a litter were killed at 3-day intervals, from 27 to 54 days of age, inclusive. At the completion of the series each 3-day group consisted of five animals.

In order to check the results of the first series with animals selected at a different season of the year, a second collection of radii was made during November and December, 1936. The selection of animals was essentially the same as in the first series except that litters were reduced to eight members each between the third and sixth days and no litters of less than eight were used. Hence one animal from each of five litters was available for each age group for the first 24 days. For the period from 27 to 54 days of age a sufficient number of litters of eight rats each was divided between the two rations so that litter mates on each ration could be killed at intervals throughout the period and provide groups of five animals each at 3-day intervals.

RESULTS AND DISCUSSION

The radii were split, stained with silver nitrate and photographed (Taylor, Klein and Russell, '38). In order to conserve space, a typical bone has been selected from each group of the first series and the bones, arranged in chronological order, are shown in figure 1. At 3 days of age calcification had not appeared in the head of any of the radii, at 6 days of age it was observed in some cases but at 9 days it was present in all members of the group. In the normal animals there was a progressive narrowing of the epiphyseal cartilage with increase in age but an immediate and rapid widening of the cartilage occurred as soon as animals were placed on the

rachitogenic ration. At about 30 days of age, after 6 days on the rachitogenic ration, the bones of the animals of this group began to differ in appearance from those of the normal. The diameter of the shaft tended to remain constant, whereas the head of the bone continued to enlarge. In the normal bones both the head and the shaft continued to grow. This difference in shaft growth gives the heads of the bones of

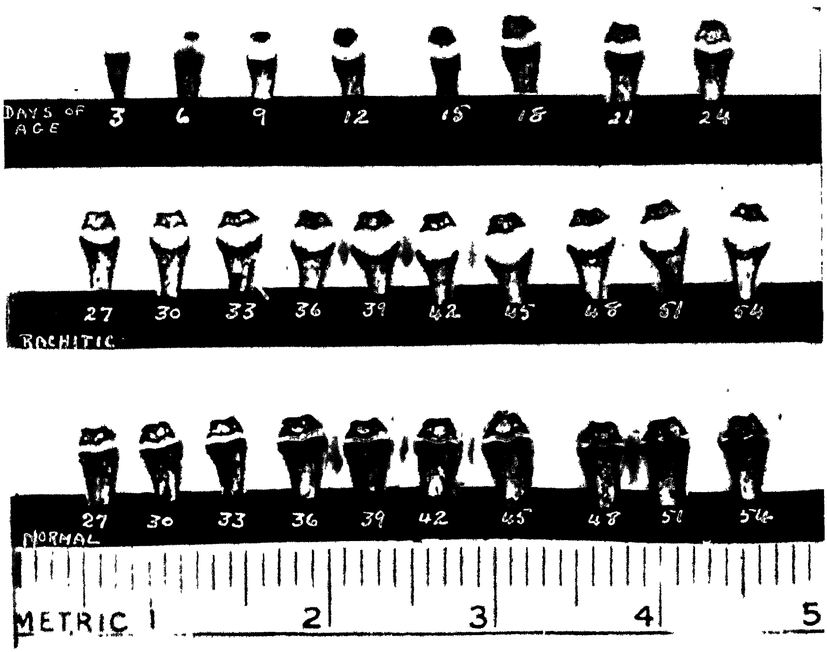

Figure 1

the rachitic series the appearance of having greater width than those of the normal, but measurement shows that the head diameter is essentially the same in the two types of bones.

The growth curves (fig. 2) reveal the marked slowing of growth which took place when the animals were placed on the rachitogenic ration. The gain in weight of animals of this colony during the depletion period (24 to 47 days of age for the preparation of animals for vitamin D assay) varies, and the gain made by the animals of this experiment, about

10 gm., was lower than the 15 to 20 gm. gain frequently observed. Even when the best growth was shown during the depletion period it was markedly less than during the same age period on the colony ration. This sharp change in growth rate indicates that the rachitogenic ration is deficient in certain factors essential for growth and that the storage of these factors is very small. The apparent retardation of the growth rate, immediately after 24 days of age, as shown by the

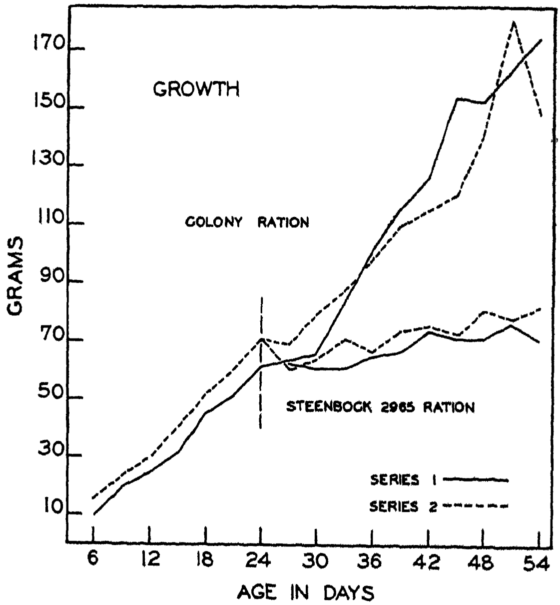

Figure 2

curves for the stock colony animals was probably due to the fact that during the first 24 days litters were progressively reduced so that the remaining rats had a better opportunity for nursing and, therefore, gained more rapidly, whereas animals from whole litters weaned at 24 days were used in obtaining the points on the growth curves for 27 days of age and subsequent ages.

The width of the epiphyseal cartilage has been used as an index of calcification. To make this measurement, the bone

photographs were enlarged so that the diameter of the photograph actually used for the measurement was ten times that of the bone itself. Width values are an average of measurements made at each side and at the center of the cartilage.

Variation of the width of the epiphyseal cartilage with age is shown in figure 3. The width of the cartilage decreased

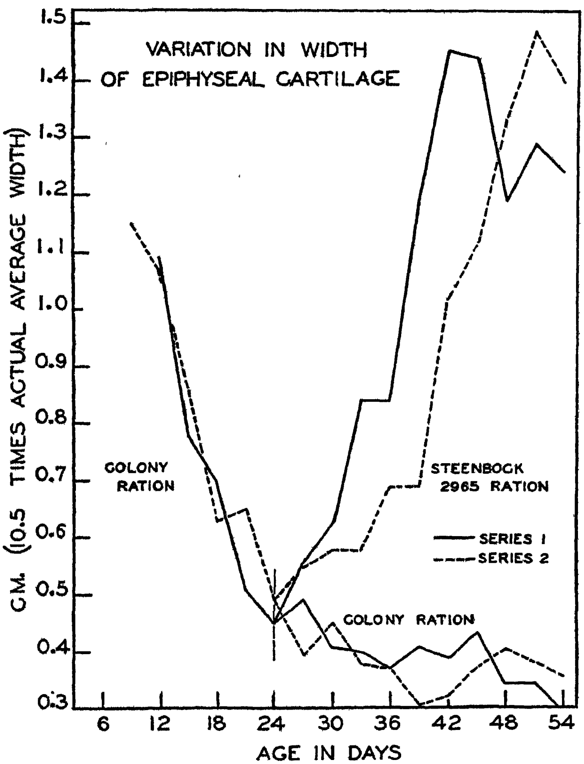

Figure 3

rapidly during the first 24 days of life. Beyond this period the decrease continued in the case of the animals placed on the colony ration but at a slower rate. Immediately after the animals were placed on the rachitogenic ration a rapid widening of the epiphyseal cartilage ensued. In series 1 a maximum width was reached at 42 days of age, which is 18 days

after the start of the depletion period. After the peak was reached, there were fluctuations in width values until the end of the experiment at 54 days of age but no further increase. This observation is in agreement with the experience of this laboratory that when rickets resistance occurs the continuance of animals beyond the usual depletion period does not induce more severe rickets.

Maximum cartilage width was apparently reached at 51 days of age, after 27 days of depletion, in series 2. The practice in this laboratory is to start animals on the assay period for vitamin D assays after 23 days of depletion on the rachitogenic ration. Had the animals of series 1 been used for assay at 47 days of age (after 23 days of depletion) the maximum epiphyseal width would have been passed but in series 2 the maximum would not have been reached. Therefore, it is conceivable that for series 2, in which the depletion process was still in progress, a different number of units of vitamin D would be necessary for a given degree of line calcification than for series 1 in which depletion had already reached a maximum. If this condition exists it may explain the wide differences in response which are sometimes observed among the animals of an assay group which have received identical quantities of vitamin D.

SUMMARY

Radii were obtained from albino rats in a stock colony ration at 3-day intervals for the age period of 3 days to 54 days, inclusive. From 24 to 54 days of age, inclusive, rats from the stock colony were fed the Steenbock rachitogenic ration 2965 and the radii taken at 3-day intervals.

The split, stained radii were photographed and the average width of the epiphyseal cartilage measured. This value for the stock colony animals decreased continuously and tended to become constant after 36 days of age. The narrowing of the epiphyseal cartilage ceased within 3 days after the animals were placed on the rachitogenic ration and a widening process

began which continued for 18 to 27 days (42 to 51 days of age) after which there was no further widening of the cartilage.

There was a marked retardation of the growth rate immediately after the animals were placed on the rachitogenic ration.

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FACTORS AFFECTING MANGANESE UTILIZATION IN THE CHICKEN

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Numerous workers have found that high levels of calcium and phosphorus in the diet of chickens increase the incidence of perosis. Repeated observations on the aggravation of perosis with sodium phosphate and steamed bone meal as well as statistical studies (Milby, '33; Hammond, '36), have placed the responsibility for this effect upon phosphorus. However, other evidence indicates that this causative effect is not due to phosphorus alone. A review of the literature has shown that phosphorus generally aggravates perosis only when a high level of calcium is also in the diet. Hunter ('31), Payne ('32), Wilgus ('37 a) and their co-workers have found that perosis can be aggravated by a high-calcium diet. On the other hand, perosis is not aggravated by a low-calcium high-phosphorus diet (Schaible et al., '33; Milby, '34; Clifcorn et al., '38).

Feeding large amounts of calcium and phosphorus induces perosis by increasing the requirement for manganese (Caskey and Norris, '38; Schaible et al., '38; Wiese et al., '38 a). Since there is no pronounced change in blood calcium and phosphorus in perosis (Hall and King, '31; Payne et al., '32; Herner and Robinson, '32; Holmes et al., '33; Heller and Penquite, '37; Wilcke, '33; Wiese et al., '38 b), and since manganese injected intraperitoneally or subcutaneously is effective in much smaller quantities than that given in the diet (Lyons, '38; Schaible, '38; Caskey, '38; Weise, '38 b and their co-workers), it seems probable that the effect of excess

calcium and phosphorus takes place in the digestive tract instead of within the body proper. Schaible ('35) has found that calcium phosphate tends to dissolve in the upper digestive tract and to reprecipitate in the lower intestines. We believed this precipitation might result in a removal of soluble manganese, thus causing perosis by creating a virtual manganese deficiency. Proceeding on this hypothesis, further evidence that phosphorus alone is not causative has been obtained and the mechanism by which calcium and phosphorus

TABLE 1

Basal diets used in growth trials

INGREDIENTS	EXPERIMENT NUMBER		
	1	2	3
%	%	%	%
Ground yellow corn	69.3	61.2	51.2
Dried buttermilk	10.0	10.0	..
Dried skim milk	..	..	20.0
Sardine meal	15.0	15.0	..
Soybean oil meal	..	..	20.0
Alfalfa leaf meal	5.0	5.0	..
Sodium chloride iodized	0.5	0.5	1.0
Reenforced fish oil (400 D)	0.2	0.3	0.3
Pulverized oystershell	..	..	0.5
Steamed bone meal	..	..	2.0
Total	100.0	92.0	95.0
Ground yellow corn and supplement	..	8.0	5.0

in the diet increase the incidence of perosis has been investigated.

GROWTH TRIAL

Observations on the effect of phosphorus from different sources on the incidence of perosis constitutes the first part of this report. A series of three growth trials was run with White Plymouth Rock chicks. The experimental procedure was essentially the same as that previously used (Wilgus et al., '37 a). The basal diets used in each of the three trials are given in table 1. The supplements added to the diets and the analyses of the final diets are given in table 2. In experiments 2 and 3, the feed containing phosphoric acid was stored

TABLE 2

Growth trials

PEN NO. SUPPLEMENT	EXPERIMENT 1					EXPERIMENT 2					EXPERIMENT 3					
	Ca	P	Mn	Weight at 6 weeks	Perosis	Ca	P	Mn	Weight at 6 weeks	Perosis	Ca	P	Mn	Weight at 6 weeks	Perosis	Mn per kilogram bone[1]
	%	*%*	*ppm*	*gm.*	*Degree*	*%*	*%*	*ppm*	*gm.*	*Degree*	*%*	*%*	*ppm*	*gm.*	*Degree*	*mg.*
1. None	0.74	0.70	10	565	10	1.21	0.85	10	299	41	1.27	0.82	13	488	16	2.8
2. St. Bone Meal	..	..	..	..	..	2.13	1.27	10	257	48	2.19	1.24	13	474	36	2.6
3. $MnSO_4 \cdot 2H_2O$	..	..	..	..	..	2.13	1.27	60	333	0	1.27	0.82	63	561	0	5.0
4. H_3PO_4	0.74	1.02	10	567	1	1.21	1.30	10	266	33	1.27	1.27	13	499	10	3.2
5. $CaCO_3$	..	..	..	..	..	2.11	0.85	10	303	45	2.17	0.82	13	466	13	3.2
6. Na_2CO_3	0.74	0.70	10	543	14	..	..	..	..	..	1.27	0.82	13	549	9	3.2
7. $H_3PO_4 + CaCO_3$	..	..	..	..	..	2.11	1.27	10	266	53	2.17	1.27	13	518	25	3.1
8. $H_3PO_4 + Na_2CO_3$	0.74	1.02	10	574	2	..	..	..	..	..	1.27	1.27	13	510	3	3.6
9. $NaH_2PO_4 \cdot H_2O$ $FeC_6H_5O_7 \cdot 3H_2O$	0.74	1.04	10	499	18	..	..	..	..	..	..	..	..	..	..	..
10. 0.17%, dry	0.74	0.70	10	542	25	..	..	..	..	..	..	..	..	..	..	..
11. 0.17%, wet	0.74	0.70	10	526	32	..	..	..	..	..	..	..	..	..	..	..
	15 chicks per pen					12 chicks per pen					20 chicks per pen					

[1] Fat-free dry bone.

in cans lined with cotton sacks and the galvanized feeders and grills were heavily coated with bakelite varnish.

The results of these growth trials are given in table 2. In all three experiments, phosphoric acid added alone to make the total phosphorus between 1.0 and 1.3% in a basal diet containing from 0.74 to 1.27% of calcium not only failed to aggravate perosis but actually appeared to alleviate the symptoms somewhat. The addition of sodium carbonate in experiments 1 and 3 alone, or with phosphoric acid in an amount to approximate monosodium phosphate, had no effect. The C.P. monosodium phosphate used in experiment 1, presumably as a standard of comparison for the production of perosis, failed likewise to be markedly aggravating, although more so than the basal diet alone. This sample of monosodium phosphate depressed growth to quite an extent but did not show any significant impurities on later spectrographic examination.

Raising the calcium content of the diet to about 2.1% with calcium carbonate did not aggravate perosis to the anticipated extent at a phosphorus level of about 0.8%. This might be expected in experiment 2, in which the severity of perosis was already great on the basal diet alone. The ineffectiveness in experiment 3 could be accounted for only if the small amount of manganese found on this salt (8 p.p.m.) was more effective when fed combined with the salt rather than alone. Discrepancies due to manganese contamination have been observed before (Wilgus et al., '37 b; Schaible et al., '38). However, when the calcium and the phosphorus levels were increased simultaneously to about 2.1% and 1.3% respectively, the incidence of perosis was enhanced. In view of the ineffectiveness of phosphorus increment alone, calcium is essential to increase the incidence of perosis.

Ferric citrate was added to the diet in experiment 1 to clarify previous conflicting results (Wilgus et al., '37 b) obtained on this material. These data definitely showed that this salt was causative.

Manganese was determined in the combined tibiae, femora and metatarsi of the left legs of five birds from each pen in experiment 3. The data show a very low concentration of manganese in all pens, although the manganese supplementation slightly increased the concentration of that element in the bones. However, this amount while somewhat higher than that reported by Gallup and Norris ('38) and by Schaible et al. ('38), is so small as to suggest that manganese is not a component part of bone structure.

IN VITRO TRIAL

Since the results of the growth trial supported the hypothesis advanced, further experiments were carried on in the laboratory. A solution of calcium nitrate was added to a solution of phosphoric acid and manganous sulfate. Concentrations of calcium, phosphate and manganous ions were similar to those found in the diets used in pens 1 and 7 of experiment 3. At a slightly acid reaction to simulate the pH of the lower digestive tract, the system was heated to boiling, cooled, the calcium phosphate precipitate removed by filtration and the precipitate washed. Manganese determinations showed the removal of all manganese from solution by the calcium-phosphate precipitate.

A similar experiment was performed with concentrations of iron and manganese similar to those used in pens 10 and 11 of experiment 1, except that ferric chloride was used. The solutions were boiled with the addition of a few cubic centimeters of n/10 NaOH to facilitate the flocculation of ferric hydroxide. On filtering and washing, the manganese was recovered in the precipitate.

These two series of experiments supported the hypothesis that the availability of manganese in the digestive tract of the chicken is decreased by an excess of calcium and phosphorus or by excess iron through adsorption or combination with the insoluble salts formed. As a final test, a feeding trial was conducted.

IN VIVO TRIAL

Three groups of four White Leghorn pullets approximately 18 weeks old and of about the same weight were placed on the following diets:

Pen 1—Perosis basal diet (experiment 3), 0.96% calcium, 0.80% phosphorus.

Pen 2—Basal diet plus 50 ppm. manganese; 0.96% calcium, 0.80% phosphorus.

Pen 3—Basal diet plus 50 ppm. manganese plus 7% steamed bone meal; 2.92% calcium, 1.65% phosphorus.

At the end of 10 days, the birds were sacrificed and contents of the crop, proventriculus and gizzard, duodenum,

TABLE 3

Solubility of manganese in the digestive tract

	TOTAL MANGANESE			DIALYZABLE MANGANESE		
	1 Basal	2 Mn	3 Mn + St.B.M	1 Basal	2 Mn	3 Mn + St.B.M
	mcg	*mcg*	*mcg*	%	%	%
1. Feed	346	1554	1090	29	38	22
2. Crop	141	1250	917	40	38	26
3. Proventriculus and gizzard	96	137	277	74	71	53
4. Duodenum	63	40	38	84	63	80
5. Small intestine						
a. Upper half	188	1086	471	61	80	32
b. Lower hlaf	229	1002	872	41	38	12
a + b	417	2088	1343	50	60	19
6. Colon	20	83	102	100	100	80
7. Caeca	45	191	128	78	0	12
Total 4 to 7 inclusive	545	2402	1611	58	57	24
8. Excreta	137	5156	4173	5	15	3

upper half of the small intestine, lower half of the small intestine, colon and caeca were removed separately. Samples of each diet and of excreta were also used. The pH of each sample was determined immediately on removal. The samples from the individuals in each pen were then combined in each of the seven fractions and poured into cellophane tubes. About 18 hours elapsed between these two steps; hence, the pH on the combined fractions were redetermined just previ-

ous to transfer. The samples were then dialyzed against distilled water for 3 days, with six changes of water.

The combined diffusates for each fraction were evaporated to dryness with about 10 cc. of HNO_3 and ashed. Total manganese was then determined by the periodate method.

The data thus obtained are summarized in table 3. They show that the presence of excess calcium and phosphorus in the diet definitely depressed the solubility of manganese in the digestive tract. This appeared to be true even in the feed,

TABLE 4

pH of feed, digestive material and excreta

MATERIAL	FRESH MATERIAL			18 HOURS LATER		
	1 Basal	2 Mn	3 Mn + St.B.M	1 Basal	2 Mn	3 Mn + St.B.M
1. Feed	6.5	6.4	6.5	..	..	..
2. Crop	4.5	5.2	4.8	4.4	4.4	4.2
3. Proventriculus and gizzard	3.5	3.3	3.3	4.0	3.8	4.1
4. Duodenum	6.0	6.2	6.0	5.3	5.6	5.7
5. Small intestine						
a. Upper half	6.1	6.3	6.3	5.6	5.5	5.8
b. Lower half	7.2	7.3	7.4	5.5	5.6	6.0
6. Colon	6.4	6.9	6.9	5.3	5.2	5.8
7. Caeca	5.5	6.5	6.1	5.4	6.6	6.5
8. Excreta	..	..	..	5.5	5.2	5.2

in the crop and in the proventriculus and gizzard. The figures are particularly impressive in the lower half of the small intestine and in the excreta. The small amount of material and of manganese in the duodenum, colon and caeca render data for these portions of less significance.

As shown in table 4, the pH of the materials decreased with standing at room temperature. Since this would tend to increase the availability of manganese, it seems likely that even more striking results would have been obtained if this shift in pH could have been avoided.

DISCUSSION

These findings strongly indicate that the perosis-aggravating action of such supplements as steamed bone meal and calcium phosphate is due to the removal of manganese from solution by the excess insoluble calcium phosphate in the intestines. The same explanation may hold for the increase in perosis following the addition of calcium salts without providing additional phosphorus, since the excess of calcium in the digestive tract would favor the formation of insoluble calcium phosphate. Likewise, when phosphorus supplementation is made to high calcium diets, as is usually the case, the formation of insoluble phosphate would be facilitated by the increased amount of both phosphorus and calcium in the digestive tract.

A calculation of the amounts of manganese injected by Lyons ('38), Schaible ('38), Gallup ('38) and Wiese ('38 b) and their co-workers into approximate concentration in the diet shows that from 6 to 10 p.p.m. was thus injected. The minimum protective levels of manganese required to be added to the basal diets for comparable protection was shown to be from 25 to 30 p.p.m. by Insko ('38), Gallup ('38 a) and Schaible ('38) and their co-workers. This indicates that not more than 20 to 40% of the manganese is available when added to the diet in these amounts. These percentages, calculated on the basis of assumed feed consumption, are comparable to those given in this report for the small intestines.

The stimulation of perosis by a small addition of ferric citrate to the diet may be explained by the formation of ferric chloride in gastric digestion and by hydrolysis to insoluble ferric hydroxide in the less acid intestines. This reaction would tend to remove the manganese in the manner demonstrated in vitro.

It is possible that the action of calcium phosphate in the digestive tract may prove to be the same mechanism that increases the iodine requirement (Thompson, '36) and inter-

feres with iron assimilation (Kletzien, '38) through the feeding of excess calcium.

The fact that manganese occurs in such a limited concentration in fat-free-dry bone is in agreement with the report of Gallup and Norris ('38) and of Schaible et al. ('38) and would seem to indicate that this element is not a component part of bone structure. The evidence brought forth by Wiese et al. ('38 b) that bone phosphatase is subnormal in active perosis and the report that manganese catalyzes phosphatase activity (Thannhauser et al., '37) might lead one to conclude that the effect of manganese is the result of its catalytic effect on phosphatase. The low serum phosphoric ester reported by these workers may be a result of low serum phosphatase, as this enzyme acts reversibly.

However, the primary effect of manganese in bone growth may be found to relate more closely to bone cartilage than to bone salt deposition. The well-known fact that the ash, calcium and phosphorus content of bone is normal in perosis and the incidence of chondrodystrophy in chick embryos from hens on a low-manganese diet (Lyons and Insko, '37) support this possibility.

SUMMARY

The finding that phosphorus supplementation to a perosis-producing diet did not aggravate perosis indicates that phosphorus per se is not a causative factor. Excess calcium in the diet is apparently essential for the stimulation of perosis.

It has been shown that insoluble calcium phosphate can remove manganous ions from solution. The decrease in diffusible manganese in the intestinal contents of chicks which was observed after feeding steamed bone meal is apparently due to this action of calcium phosphate.

Ferric hydroxide also removed manganese from solution. Ferric citrate in the diet increased the severity of perosis.

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THE UTILIZATION OF ENERGY PRODUCING NUTRIMENT AND PROTEIN AS AFFECTED BY THE LEVEL OF THE INTAKE OF BEEF MUSCLE PROTEIN [1]

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FOUR FIGURES

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In 1935 and 1937 papers were published from this laboratory (Forbes, Swift, Black and Kahlenberg, '35; and Forbes, Voris, Bratzler and Wainio, '37) setting forth results of experiments on the plane of protein intake as affecting the utilization of food energy and protein, the main nitrogenous component of the diets having been casein.

These experiments were in excellent agreement in revealing moderate decrease in heat production with increase in the protein content of equicaloric diets containing 10, 15, 20, 25, 30, 35 and 45% of protein. However, certain questions remained as to whether the facts characterizing casein in this connection are true of protein in general, and also as to the influence of the plane of food intake in the same relations.

The experiments to be discussed, therefore, were designed to contribute to the answer of these two questions.

The procedure was the same as in the earlier studies of the same general subject; that is, the body gains of energy and of protein were determined by difference between values for the body components of a control group representing the experimental subjects at the beginning of the investigation and

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parallel values for subjects at the end of the 70-day experiment; and the heat production was measured as the gross energy of the food minus the energy of the excreta and of the body gain for the entire period.

As in the earlier experiments the rats were fed with quadruplet food control. The protein contents of the diets were 10, 25, 35 and 45%, respectively, each rat of a quadruplet receiving a different intake of protein, but the same intake of energy.

The twelve quadruplets of rats serving as experimental subjects were divided into two groups of six quadruplets each, one of which was held to approximately the same food intake as that which prevailed in the earlier experiments with casein, while the other was permitted to consume food at a much higher rate, determined for each quadruplet by the ad libitum consumption of the poorest eater in each quadruplet. The average consumption of food energy in this latter group of six quadruplets was 44.7% higher than the average in the earlier experiments.

The composition of the diets as given in table 1 requires no comment.

In table 2 it is shown that the higher plane of food intake led to the larger body gains at each percentage of protein—which is virtually inevitable.

The relative efficiency of the diets to produce gain in body weight is indicated in figures 1 and 2. These graphs represent gross body weight.

The nitrogen and the fat gained, and the quantitative relation of the one to the other, are determined as resultants of a complication of factors, to wit—the amounts and proportions of protein and non-protein nutriment 1) in the diets, 2) required for maintenance, and 3) available for body increase.

At the lower plane of food intake the comparatively low values for both nitrogen and fat gained only suggest intake less than the optimum. The rapid decrease in fat gained with increasing order of protein contents of the diets implies di-

TABLE 1

Composition of diets

	10% PROTEIN RATION	25% PROTEIN RATION	35% PROTEIN RATION	45% PROTEIN RATION
	%	%	%	%
Cellu flour	4.00	4.00	4.00	4.00
O. and M. salt mixture	4.00	4.00	4.00	4.00
NaCl	1.00	1.00	1.00	1.00
Butterfat	1.00	1.00	1.00	1.00
Yeast [1]	6.00	6.00	6.00	6.00
Dextrin	62.34	49.29	40.59	31.89
Beef protein	8.19	26.55	38.80	51.04
Crisco	13.47	8.16	4.61	1.07
	100.00	100.00	100.00	100.00

[1] The yeast mixture contained 5 parts of brewer's yeast to 1 part of irradiated yeast.

Note: Carotene, in oil, was added to each diet in the proportion of 0.333 gm. per kilogram. Each diet contained approximately 1.4 U.S.P. units of vitamin A per gram.

TABLE 2

Food eaten and average quantities, character and gross efficiency of gains in weight during 70 days

PER CENT OF PROTEIN IN DIET	INITIAL LIVE WEIGHT	FINAL LIVE WEIGHT	FOOD EATEN (DRY MATTER)	GAIN IN EMPTY BODY WEIGHT	FOOD (DRY MATTER) PER GRAM BODY GAIN	NITROGEN OF BODY GAIN	FAT GAINED	FAT GAINED PER GRAM N GAINED
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Lower food intake								
10	48	123	430	76	5.7	2.5	12.60	5.0
25	48	141	430	94	4.6	3.5	8.35	2.4
35	48	139	430	91	4.7	3.4	7.07	2.1
45	48	132	429	83	5.2	3.2	4.31	1.3
Higher food intake								
10	49	173	630	121	5.2	3.7	24.31	6.6
25	50	218	630	167	3.8	5.5	29.61	5.4
35	50	220	630	168	3.8	5.8	22.81	3.9
45	50	207	629	154	4.1	5.2	23.91	4.6

Note: All data in all tables are averages per rat, from groups of six on the same dietary treatment, for 70 days.

minishing non-nitrogenous nutriment; and the associated gain in body nitrogen, from the 10% protein to the 25% protein diet, followed by a decrease in gain in nitrogen from the 25% protein to the 45% protein diet, seems to be due primarily to the fact that the protein was unaccompanied by enough non-protein nutriment to provide for its efficient utilization.

At the higher plane of nutrition the amounts of nitrogen and of fat gained were inevitably much higher than at the lower plane, with the best conditions for gain of nitrogen

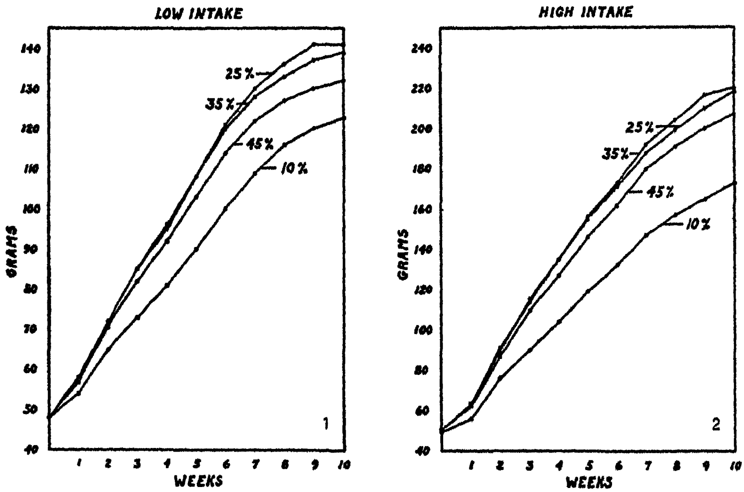

Fig. 1 Shows average gains in body weight of groups of six growing rats as affected by the plane of intake of beef muscle protein in equicaloric diets consumed at a restricted rate.

Fig. 2 Shows average gains in body weight of groups of six growing rats as affected by the plane of intake of beef muscle protein in equicaloric diets consumed at a relatively liberal rate.

with the 35% protein diet and for gain of fat with the 10% protein diet.

It is clear, therefore, that the character of the body increase as to proportion of fat to protein differs in accord with character of the food.

The distribution of the food energy as affected by the plane of intake and by the percentage of protein in the equicaloric diets is shown in table 3 and in figures 3 and 4.

TABLE 3

Distribution of average total food energy[1] as affected by the plane of protein intake

PER CENT OF PROTEIN IN DIET	FOOD ENERGY	FECES	DI-GESTED	URINE	METABO-LIZED	BODY GAIN	BODY GAIN AS PROTEIN	BODY GAIN AS FAT	HEAT
	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.
Lower food intake									
10	2138	216	1922	42	1880	210	91	119	1670
25	2138	186	1952	98	1854	201	122	79	1653
35	2138	178	1960	142	1818	187	120	67	1631
45	2138	187	1951	183	1768	156	115	41	1612
Higher food intake									
10	3136	339	2797	58	2739	364	136	228	2375
25	3136	296	2840	142	2698	480	201	279	2218
35	3136	279	2857	209	2648	423	208	215	2225
45	3136	298	2838	261	2577	415	189	226	2162

[1] For 70 days.

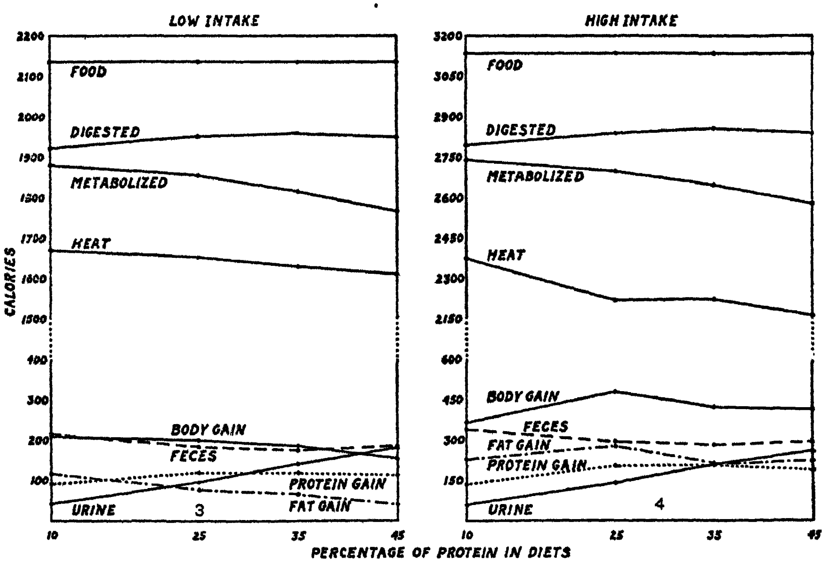

Fig. 3 Illustrates the distribution of food energy by groups of six growing rats as affected by the plane of intake of beef muscle protein in equicaloric diets consumed at a restricted rate.

Fig. 4 Illustrates the distribution of food energy by groups of six growing rats as affected by the plane of intake of beef muscle protein in equicaloric diets consumed at a relatively liberal rate.

The more notable facts in this relation, at the two planes of food intake, were 1) that the digestibility of the nutrients containing the food energy increased slightly from the 10% to the 35% protein diets, and then decreased very slightly between the 35% and the 45% protein diets; 2) that the urine energy increased markedly and regularly from the 10% to the 45% protein diet; 3) that the metabolizability of the diets diminished at a nearly regular rate from the 10% to the 45% protein diet; 4) that the heat production diminished slightly from the 10% to the 45% protein diet, at a rate less than the accompanying decrease in metabolizable energy at the lower plane of nutrition, but greater than the decrease in metabolizable energy at the higher plane of nutrition; 5) that the body gain diminished materially at the lower plane of intake and increased slightly (with some irregularity) at the higher plane of food intake, from the 10% to the 45% protein diet.

The facts in relation to protein gain and fat gain are presented but are less notable. As indicative of the accuracy of the experimental work, the average recovery of food nitrogen in excreta and in body increase was 98.5%.

The method of the experiment did not provide an analysis of the heat production, though in the earlier experiments on the same subject it was demonstrated that differences in the protein content of the equicaloric diets did not affect the basal metabolism.

The comparative effects of casein and beef muscle protein on the distribution of the food energy may be observed from the data relating to the rats on the lower food intake (table 3), and from parallel data in the 1937 paper of Forbes, Voris, Bratzler and Wainio (the lower half of table 3, p. 293).

The food energy being essentially the same in the rations containing the casein and the beef muscle, the casein ration was somewhat more efficiently digested. Urine energy values were almost identical. Metabolizable energy values, therefore, differed as the digestible energy values differed.

Values for energy of body gain were higher for the casein; as to heat production, the values differed but little—between

1699 to 1576 for the casein ration and 1670 to 1612 for the beef protein ration.

These data, therefore, reveal no evidence whatever of a greater heat stimulating effect of beef muscle protein than of casein for growing rats.

The metabolizability of the food energy is obviously a factor of importance in relation to the heat production, but that this factor alone did not control the heat production is indicated by the facts that at the lower plane of nutrition the metabolizable energy diminished about twice as much as did the heat production, from the 10% and the 45% diets, while at the higher plane of nutrition the heat production diminished more rapidly than did the metabolizable energy, with increase in the protein content of the equicaloric diets.

As in the earlier experiments on the same general subject, metabolizable energy was computed as that of the food minus that of the excreta without correction for the non-metabolizable portion of the energy of the body increase.

The reason that this correction was not made is that it would have had the effect to obscure the values of the diets for growth—an important consideration in the present investigation.

The distribution of food nitrogen, as accounted for in table 4, shows that the digestibility of the nitrogen increased slightly but consistently and the urine nitrogen prominently, with increase in the protein content of the diets.

At both planes of nutrition, with increasing order of protein contents of the diets the grams of food nitrogen retained first increased materially and then decreased slightly; while the percentage of the food nitrogen retained was highest with the 10% protein diet and diminished to less than one-third of this maximum proportion with the 45% protein diet.

That the calories per gram of nitrogen in the urine, as set forth in table 5, invariably decreased in accord with progressively greater protein contents of the diets signifies a decrease in the proportion of nitrogenous compounds of high energy value per gram of nitrogen.

TABLE 4

Distribution of average total food nitrogen as affected by the percentage of protein in the diet

PER CENT OF PROTEIN IN DIET	FOOD NITROGEN	FECES NITROGEN	DIGESTED NITROGEN		URINE NITROGEN		BODY GAIN OF NITROGEN	
	gm.	*gm.*	*gm.*	*%*	*gm.*	*%*	*gm.*	*%*
Lower food intake								
10	7.3	1.0	6.3	86.3	4.0	54.8	2.5	34.2
25	18.2	1.4	16.8	92.3	12.7	69.8	3.5	19.2
35	25.5	1.8	23.7	92.9	19.8	77.6	3.4	13.3
45	32.4	2.1	30.3	93.5	26.4	81.5	3.2	9.9
Higher food intake								
10	10.7	1.6	9.1	85.0	5.3	49.5	3.7	34.6
25	26.7	2.3	24.4	91.4	17.9	67.0	5.5	20.6
35	37.3	2.9	34.4	92.2	28.0	75.1	5.8	15.5
45	47.5	3.4	44.1	92.8	38.0	80.0	5.2	10.9

TABLE 5

Relationship of nitrogen to energy in urine as affected by the proportion of the protein in the diet

PER CENT OF PROTEIN IN DIET	NITROGEN OF URINE	ENERGY OF URINE	ENERGY PER GRAM OF NITROGEN IN URINE
	gm.	*Cal.*	*Cal.*
Lower food intake			
10	4.0	42	10.6
25	12.7	98	7.7
35	19.8	142	7.2
45	26.4	183	6.9
Higher food intake			
10	5.3	58	10.9
25	17.9	142	7.9
35	28.0	209	7.5
45	38.0	261	6.9

There is in the results of this experiment, therefore, no evidence whatever of an increase in heat production associated with progressively greater protein contents of the equicaloric diets; in fact the findings were in unmistakable agreement with all of the previous observations showing that with higher percentages of protein in the diets there is decrease in heat.

In seeking to explain this result the mind naturally turns to the metabolizability of the diets and to the basal metabolism and the voluntary activity.

In regard to the metabolizable energy of the diets, it has been shown that at the 10, 15 and 20% protein levels there is no decrease in metabolizable energy with increasing protein contents of the diets, though the heat production diminishes materially at these levels. Whatever was the influence of the diminishing metabolizability of the diets on the heat production at the higher protein levels, this factor cannot be regarded as the dominant one.

Basal metabolism was not determined in connection with the present experiment, but in the previous studies of the same subject covering the same range of percentages of dietary protein no change in the basal metabolism was observed.

In all probability a special investigation of this aspect of the matter would be required for the complete establishment of the facts, since the decrease in heat with increase in protein was not particularly extensive, and the factoring of this decrease could readily go wrong in any except a thorough investigation of that particular matter. It is, therefore, probably wise to hold in abeyance a decision as to whether the demonstrated decrease in heat production coincident with increasing percentages of dietary protein signifies decrease in basal metabolism, or in voluntary activity, or in both, though the evidence now at hand indicates that the decrease is in the voluntary activity.

Further light will be thrown on the whole problem of the present investigation by experiments now in progress with mature animals, which cannot store much protein, as subjects.

This investigation should reveal the influence on the heat production of protein which is utilized for fat or for energy production.

SUMMARY

The influence of progressively greater protein contents of equicaloric diets was studied by means of feeding, metabolism and body analysis experiments with two groups, twenty-four each, of albino rats as subjects with quadruplet food control, the principal food protein being from beef muscle.

One group was limited in food consumption to the approximate amount eaten in earlier experiments with casein as the principal food protein, and the other was allowed to eat 44.7% more.

The most notable observations relating to the distribution of food energy were: 1) that the metabolizability of the diets diminished at a nearly regular rate from the 10% to the 45% protein diet; 2) that the heat production diminished slightly from the 10% to the 45% protein diet at a rate less than the accompanying decrease in metabolizable energy at the lower plane of nutrition, but greater than the decrease in metabolizable energy at the higher plane of nutrition; 3) that the body gain diminished materially at the lower plane of intake and increased slightly at the higher plane of food intake from the 10% to the 45% protein diet.

The distribution of the food nitrogen and the composition of the body increase, as affected by the composition of the diets, were also observed.

By comparison with earlier work it is shown that beef muscle protein does not have a greater heat stimulating effect than casein for growing rats.

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DYNAMIC EFFECTS AND NET ENERGY VALUES OF PROTEIN, CARBOHYDRATE AND FAT [1]

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This investigation, with growing albino rats as subjects, was based on the following fundamental conceptions: 1) that a nutrient is never, under any conditions, metabolized by itself—regardless of whether it is of body origin, as during fast, or is fed by itself to a subject in the postabsorptive state, or is superimposed upon a maintenance diet; 2) that the dynamic effect of a food substance depends on the amount and kind of nutriment with which it is utilized and on the length of time since food was received; and 3) that the conditions under which any nutrient possesses its maximum, normal nutritive value—the logical conditions, therefore, for the measurement of dynamic effects and net energy values—are as in complete and perfect diets or rations (Forbes, '33).

These conceptions may be considered as logical consequences of the ideas of Rubner ('02) who understood that the energy expense of utilization of a mixed diet is less than the sum of such values for the components of the diet, and who also understood the influence of the sparing of body nutrients by food nutrients fed at planes of nutrition below energy equilibrium in relation to the apparent dynamic effect of the food nutrients.

Many followers of Rubner, and later of Lusk, have also subscribed to these ideas from time to time in various relations, but have not followed them consistently in the determination of dynamic effects of nutrients.

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In fact, much the greater part of the literature on this subject is based on observations which the authors of this paper believe to be at least questionable and of indefinite nutritive significance, since they have been made in experiments on subjects at planes of nutrition below maintenance (usually at the so-called basal plane) in which there was, or may have been, a sparing of body nutrients by the food nutrients under investigation.

An appreciation of the facts in this situation led to the conclusion by Forbes, Braman, Kriss et al. ('30) that a heat increment determined at a plane, or between planes, of nutrition below maintenance is less than the true energy expense of food utilization by the amount of energy by which the nutrient fed has spared the katabolism of body substance.

The point of view of the Institute of Animal Nutrition of the Pennsylvania State College with reference to the measurement of specific dynamic effects, which is based on the experimental procedures of Kellner, and of Armsby, as modified since 1922 by Forbes and associates, is set forth in detail in a recent paper by Kriss, Forbes and Miller ('34).

The main features of the procedure advocated are: 1) the measurement of dynamic effects as the difference in the heat production in comparable experimental periods, with the animal established on and thoroughly adjusted to continuing nutritive regimens differing only in the amount of the component of interest, thus permitting heat measurements under such conditions and during such time that values of actual nutritive significance may be obtained; and 2) the use of the status of energy equilibrium as the base from which the heat increment is measured, instead of any condition of fast, as a means of avoiding confusion of the dynamic effect of the food nutrient with the waste heat of utilization of the body nutrients katabolized during experimental periods below the maintenance level.

Obviously the difference between this and the usual point of view of students of the subject is all-important; and, in the absence of correct understanding and general agreement

as to these considerations, the discussion of intimate details of underlying physiology and thermodynamics has very naturally led only to continuing confusion of thought.

But whatever the understanding of the fundamental facts involved in this problem, and whatever the details of experimental procedure employed, the energy expenses of food utilization in general, and the dynamic effects of specific nutrients, appear to be so much affected by so many influences having to do with nutrient combinations, planes of nutrition, and the purposes for which the nutrients are used, that if such values are to possess the significance of constants this must be by virtue of rigorously formalized definition and experimental control. The values, therefore, apply with accuracy only to the special conditions under which they have been determined.

In the investigation to be discussed determinations were made of the dynamic effects and net energy values of protein, carbohydrate and fat, singly and of the three combined, these supplements being superimposed, in all cases, upon an approximately complete, basal, maintenance diet.

It will be understood that in any determination of heat increments the entire supplementing or sparing effect of the combination of the basal with the test nutrients must be referred to the 'test nutrients' since there is no scientific method for apportioning the value of a diet among its constituents—a point of view which becomes obvious and indisputable when it is realized that inorganic salts, which possess no energy value in the usual sense, and vitamins, which contributed, at the most, negligible amounts of energy to diets, may profoundly affect the metabolism of energy producing nutrients.

Results are given of two series of experiments, each series consisting of parallel determinations on lots of five rats each, at body weights of approximately 100 and 240 gm., respectively. A different lot of five rats was used for each treatment at each age, and all data presented are averages representing five individuals.

Each series of experiments extended over several weeks' time, during spring and summer. With the advancement of the season there were naturally many changes in the climatic conditions to which the animals were subjected, and it is obvious that there were circumstances affecting the heat production which were not under control; but the results possess significance by comparison, apart from considerations as to their absolute validity.

Earlier experiments, the results of which are not presented, served to emphasize the fact that from the human point of view the life-span of the rat is indeed short, and that biological measurements on the same individual growing rats extending through a number of weeks may readily defeat their own purposes if it be assumed that the same background of physiological conditions continues throughout such an investigation.

Indeed it was found quite impracticable to maintain correct conditions for the comparison of diets by the use of the same individual animals as subjects through 6 to 10 weeks' observations. At the end of such a series of experiments the subjects were, in effect, not the same individuals as at the beginning.

A prominent difficulty encountered in this relation is that we do not have an entirely reliable means of computing the heat production of an animal to the basis of a standard live weight, as is necessary if the experimental treatment has produced changes in weight during the time required for the development of the desired response to the treatment.

Also, the previous feeding affects the heat production, and tends to confuse apparent dynamic effects in a series of observations with different diets and the same animals.

Further, rats do not eat as regularly as do some of the larger domestic animals and do not lend themselves as effectively to feeding at definite rates of intake in relation to the live weight; still the live weight does affect the heat production, and this fact must be dealt with in an effective manner.

At the expense of much loss of effort, therefore, the authors have learned that an advantageous way of providing for an extended series of dietary observations on growing rats is to select a different lot of animals of the same age and weight and having had the same preparatory treatment as the subjects for each unit of the program, and then to give them all the same quantities of feed.

Obviously, close consanguinity in the subjects and an adequate number of individuals in each experimental group are essential.

PROCEDURE

The general method of experimentation and the equipment used were approximately as described in a recent paper by Forbes, Kriss and Miller ('34), but with alterations in equipment as follows: a change of the water bath in which the respiration chamber was immersed to an air compartment with windows permitting observation of the animals; and the suspension of the respiration chamber from a work adder—thus providing effectively for the exclusion of data representing intervals of time during which there was any considerable activity of the experimental subjects.

Heat production was measured by the Haldane open-circuit respiratory quotient procedure in intervals of essentially complete rest during 7-hour periods in the respiration chamber, with the animal adjusted to and established on its dietary regime in the experiment.

Preliminary to the fasting heat measurement (determined to provide for the correction of the heat production of comparable animals to a basis of exactly the same live weight) the rats were given a maintenance ration for 5 days. Then an interval of 25 hours elapsed after the last feed was given before the actual fasting heat measurement was begun.

In preparation for the determination of the heat production from the basal and the supplemented diets the rats were thoroughly established on these planes of nutrition by preliminary feeding; then the same treatment was continued, during 1 day for the measurement of the heat production,

followed by 7 days for the collection of the excreta—for the determination of metabolizable energy.

The measurement of the heat production of a rat on the basal diet, or on the basal diet plus a supplement, was made after the animal had received at 8.00 A.M. one-half of its daily food allowance. Half an hour was allowed for eating and another half-hour for postprandial activity before the animal was placed in the respiration chamber at 9.00 A.M. The first hour's heat production was always discarded. The values for heat production actually used, therefore, represented the third to the eighth hours, inclusive, after feeding—minus periods of activity.

The experimental subjects were male, albino rats from the colony of this institute, in which a stock of a high degree of vigor and uniformity has been developed in the course of many generations of selective breeding.

The basal diets were composed of two-thirds ground wheat and one-third whole milk powder (Parlac) to which was added sodium chloride in the proportion of 0.5% of the wheat and milk powder.

The protein supplement was lean beef muscle, sterilized, dried at a low temperature, reduced to a powder and extracted with ether for 48 hours. The carbohydrate used was dextrin (Amidex) which was free from nitrogen, and the fat used was lard which was fed as purchased.

EXPERIMENTAL PROGRAM

The two experiments were conducted between March 10 and April 22, and between June 29 and August 9, 1938. The schedules of experimentation comprise tables 1 and 5, and the numerical data are presented in tables 2 to 4 and 6 to 8.

In addition to the metabolism experiments with the rats on feed, the 24-hour fasting heat production was determined as follows:

In experiment I this value was determined for the younger rats at an average age of 70 days, and average weight of

106 gm., on March 24th and 25th, this quota corrected to a standard weight of 100 gm. being 11.18 ± 0.09 kilo calories; and the fasting heat outgo was determined for the older rats at an average age of 113 days and an average weight of 237 gm., on May 6th to 12th, this value corrected to the basis of 240 gm. live weight being 19.40 ± 0.15 kilo calories.

TABLE 1

Schedule of experimentation. Experiment I

GROUP NUMBERS	DAILY FEEDING TREATMENT	AVERAGE AGE	AVERAGE WEIGHT	DATES OF RESPIRATION MEASUREMENTS
		days	*gm.*	
1	5 gm. basal diet	56	107	March 10–11
2	5 gm. basal diet + 3 gm. beef muscle protein	45	100	March 3–7
3	5 gm. basal diet + 3 gm. dextrin	46	100	Feb. 28–March 4
4	5 gm. basal diet + 1.24 gm. lard	49	101	March 3–7
5	5 gm. basal diet + 1.6 gm. mixture, b.m.p., d. and l.	45	100	March 2–5
6	9 gm. basal diet	99	238	April 22–28
7	9 gm. basal diet + 3 gm. beef muscle protein	87	240	April 12–25
8	9 gm. basal diet + 3 gm. dextrin	91	240	April 18–21
9	9 gm. basal diet + 1.24 gm. lard	92	240	April 16–26
10	9 gm. basal diet + 1.6 gm. mixture, b.m.p., d. and l.	92	242	April 16–22

TABLE 2

Utilization of nitrogen. Experiment I

GROUP NUMBERS	TREATMENTS	DAILY NITROGEN INTAKE	NITROGEN DIGESTED	NITROGEN OF URINE	NITROGEN RETENTION
		mg.	%	%	%
1	Basal diet	119	86.6	76.5	10.1
2	Basal diet + beef muscle protein	544	92.3	77.0	15.3
3	Basal diet + dextrin	124	85.5	27.4	58.1
4	Basal diet + lard	119	84.9	26.9	58.0
5	Basal diet + mixture, b.m.p., d. and l.	166	87.3	34.3	53.0
6	Basal diet	213	82.2	70.9	11.3
7	Basal diet + beef muscle protein	654	90.1	73.1	17.0
8	Basal diet + dextrin	211	78.2	47.4	30.8
9	Basal diet + lard	223	81.6	49.3	32.3
10	Basal diet + mixture, b.m.p., d. and l.	255	82.4	55.7	26.7

In experiment II the fasting heat production was determined for the younger rats at an average age of 67 days, and an average weight of 91 gm., on July 12th to 13th, this value corrected to the basis of 100 gm. live weight being 10.77 ± 0.22 kilo calories; and the same value was determined for the older rats at an average age of 93 days, and average weight of 229 gm. on August 4th to 11th, this value corrected to the basis of 240 gm. being 20.44 ± 0.18 kilo calories.

The quantities of the basal diets given were adequate for maintenance, and the supplements were fed superimposed upon these basal diets.

The quantities of the supplements given (3 gm. each of beef muscle protein and dextrin, 1.24 gm. of lard and 1.6 gm. of a mixture of the three) were such as contained approximately equal amounts of metabolizable energy; and the mixture of 62.5% lard and 18.75% each of dextrin and beef muscle protein supplied fat, carbohydrate and protein in the approximate proportions in which they are katabolized during fast.

DISCUSSION OF RESULTS

The data with reference to nitrogen utilization in tables 2 and 6 show that in both experiments and with both weights of rats, the diets supplemented by beef muscle protein, and therefore containing the maximum amounts of protein, were characterized by the maximum digestibility of their protein. These same diets led to the maximum percentage elimination of the dietary nitrogen in the urine; and among the supplements, the beef muscle protein led to the lowest percentage nitrogen utilization with both weights of rats in both experiments.

These observations are to be explained primarily by the excessive intake of protein by the rats which received the beef muscle preparation.

The digestibility of the protein of the basal diets and of the diets containing the non-nitrogenous supplements was naturally of the same general degree, and the digestibility of

TABLE 3

Metabolizable energy of diets and of supplements. Experiment I

GROUP NUMBERS	TREATMENTS	DAILY ENERGY INTAKE	ENERGY OF FECES	ENERGY OF URINE	METABOLIZABLE ENERGY	
					Total	Of supplement
		Cal.	%		%	%
1	Basal diet	21.89	9.7	4.0	86.3	
2	Basal diet + beef muscle protein	37.44	8.1	9.7	82.2	76.4
3	Basal diet + dextrin	33.31	6.5	2.7	90.8	99.2
4	Basal diet + lard	33.52	7.2	2.7	90.1	97.2
5	Basal diet + mixture, b.m.p., d. and l.	30.04	6.6	3.7	89.7	95.7
6	Basal diet	39.39	10.9	3.1	86.0	
7	Basal diet + beef muscle protein	54.50	9.3	8.1	82.6	73.9
8	Basal diet + dextrin	50.70	9.6	2.7	87.7	93.7
9	Basal diet + lard	51.40	10.0	3.3	86.7	89.0
10	Basal diet + mixture, b.m.p., d. and l.	51.61	9.4	3.7	86.9	90.1

TABLE 4

Daily heat production, dynamic effect and net energy. Experiment I

GROUP NUMBERS	TREATMENTS	DAILY HEAT PRODUCTION	DYNAMIC EFFECTS OF SUPPLEMENTS	NET ENERGY OF SUPPLEMENTS	
		Cal.	*% metabolizable*	*% metabolizable*	*Cal. per gram*
1	Basal diet	13.05 ± 0.04[1]			
2	Basal diet + beef muscle protein	21.37 ± 0.14[1]	70.1	29.9	1.19
3	Basal diet + dextrin	19.22 ± 0.21[1]	54.5	45.5	1.72
4	Basal diet + lard	16.19 ± 0.15[1]	27.8	72.2	6.59
5	Basal diet + mixture, b.m.p., d. and l.	17.63 ± 0.16[1]	39.4	60.6	4.41
6	Basal diet	23.34 ± 0.21[2]			
7	Basal diet + beef muscle protein	30.37 ± 0.15[2]	62.9	37.1	1.38
8	Basal diet + dextrin	27.97 ± 0.12[2]	43.7	56.3	1.99
9	Basal diet + lard	26.50 ± 0.17[2]	29.6	70.4	6.06
10	Basal diet + mixture, b.m.p., d. and l.	26.62 ± 0.19[2]	29.8	70.2	4.83

[1] Corrected to standard weight of 100 gm.

[2] Corrected to standard weight of 240 gm.

the protein of the diets containing the mixture of the supplements (one of them, beef protein) was slightly higher than that of the diets containing no beef protein.

The percentage retention of the dietary nitrogen was high in the periods in which the nitrogen intake was relatively low and the intake of non-nitrogenous nutriment relatively high, that is, in periods 3, 4, 5, 8, 9 and 10, in which the dextrin or the lard, or the two together, tended to spare the protein of the basal diet from katabolism for energy production.

TABLE 5

Schedule of experimentation. Experiment II

GROUP NUMBERS	DAILY FEEDING TREATMENT	AVERAGE AGE	AVERAGE WEIGHT	DATES OF RESPIRATION MEASUREMENTS
		days	*gm.*	
1	5 gm. basal diet	54	96	June 29–30
2	5 gm. basal diet + 3 gm. beef muscle protein	44	100	June 20–28
3	5 gm. basal diet + 3 gm. dextrine	48	101	June 22–25
4	5 gm. basal diet + 1.24 gm. lard	48	101	June 21–27
5	5 gm. basal diet + 1.6 gm. mixture, b.m.p., d. and l.	50	100	June 22–28
6	9 gm. basal diet	79	235	July 21–28
7	9 gm. basal diet + 3 gm. beef muscle protein	82	241	July 25–Aug. 3
8	9 gm. basal diet + 3 gm. dextrin	81	240	July 22–Aug. 3
9	9 gm. basal diet + 1.24 gm. lard	85	241	July 26–Aug. 9
10	9 gm. basal diet + 1.6 gm. mixture, b.m.p., d. and l.	83	242	July 25–Aug. 9

The data representing the metabolizable energy of the diets and of the supplements comprise tables 3 and 7.

The values for urinary energy, as given, are as corrected for nitrogen retention, that is, as though the subjects had been in nitrogen equilibrium. This is for the purpose of recognizing that a portion of the body increase of protein is non-metabolizable in the sense that it cannot be transformed into heat but, in the event that the stored substance is katabolized, is eliminated in the urine in compounds containing potential energy.

The notable points in relation to the metabolizable energy of the diets are 1) the relative constancy of the percentage of the food energy appearing in the feces, 2) the relatively high percentage of the food energy appearing in the urine in those periods in which the basal ration was supplemented with the beef muscle protein, 3) the relatively low metabolizability of the food energy in the same periods (2 and 7), and 4) among the supplements, the relatively low metabolizability of the energy of the beef muscle protein preparation.

TABLE 6

Utilization of nitrogen. Experiment II

GROUP NUMBERS	TREATMENTS	DAILY NITROGEN INTAKE	NITROGEN DIGESTED	NITROGEN OF URINE	NITROGEN RETENTION
		mg.	%	%	%
1	Basal diet	128	85.2	76.6	8.6
2	Basal diet + beef muscle protein	532	92.1	77.3	14.8
3	Basal diet + dextrin	125	85.6	38.4	47.2
4	Basal diet + lard	128	85.9	35.9	50.0
5	Basal diet + mixture, b.m.p., d. and l.	167	87.4	49.1	38.3
6	Basal diet	230	83.0	62.2	20.8
7	Basal diet + beef muscle protein	632	89.1	67.7	21.4
8	Basal diet + dextrin	226	81.0	39.8	41.2
9	Basal diet + lard	226	80.1	43.8	36.3
10	Basal diet + mixture, b.m.p., d. and l.	273	83.2	52.8	30.4

The main results of the experiments, the heat production from the diets, and the dynamic effects and net energy values of the supplements, comprise tables 4 and 8.

In seven instances among the total of eight, the dynamic effects of the supplements were higher for the 100-gm. rats than for the 240-gm. rats.

In both experiments and with both weights of rats, the dynamic effects of the individual supplements were in the same order—protein highest, carbohydrate intermediate and fat lowest.

TABLE 7

Metabolizable energy of diets and supplements. Experiment II

GROUP NUMBERS	TREATMENTS	DAILY ENERGY INTAKE	ENERGY OF FECES	ENERGY OF URINE	METABOLIZABLE ENERGY	
					Total	Of supplement
		Cal.	%	%	%	%
1	Basal diet	22.07	8.8	3.5	87.7	
2	Basal diet + beef muscle protein	36.47	7.9	9.8	82.3	74.0
3	Basal diet + dextrin	33.41	6.6	2.6	90.8	96.9
4	Basal diet + lard	33.53	6.7	2.5	90.8	96.9
5	Basal diet + mixture, b.m.p., d. and l.	33.79	6.9	3.4	89.7	93.4
6	Basal diet	39.73	10.7	4.4	84.9	
7	Basal diet + beef muscle protein	54.34	9.3	8.0	82.7	76.4
8	Basal diet + dextrin	50.74	9.3	3.4	87.3	95.6
9	Basal diet + lard	51.01	9.8	3.4	86.8	93.4
10	Basal diet + mixture, b.m.p., d. and l.	51.57	9.2	3.8	87.0	93.8

TABLE 8

Daily heat production, dynamic effect and net energy. Experiment II

GROUP NUMBERS	TREATMENTS	DAILY HEAT PRODUCTION	DYNAMIC EFFECT OF SUPPLEMENTS	NET ENERGY OF SUPLEMENTS	
		Cal.	*% metabolizable*	*% metabolizable*	*Cal. per gram*
1	Basal diet	13.27 ± 0.14[1]			
2	Basal diet + beef muscle protein	20.21 ± 0.23[1]	65.0	35.0	1.24
3	Basal diet + dextrin	17.81 ± 0.18[1]	41.3	58.7	2.15
4	Basal diet + lard	16.59 ± 0.14[1]	29.9	70.1	6.28
5	Basal diet + mixture, b.m.p., d. and l.	16.40 ± 0.06[1]	28.6	71.4	4.89
6	Basal diet	25.53 ± 0.40[2]			
7	Basal diet + beef muscle protein	31.16 ± 0.17[2]	50.4	49.6	1.85
8	Basal diet + dextrin	29.32 ± 0.13[2]	36.0	64.0	2.25
9	Basal diet + lard	28.49 ± 0.48[2]	28.0	72.0	6.12
10	Basal diet + mixture, b.m.p., d. and l.	28.53 ± 0.18[2]	26.9	73.1	5.07

[1] Corrected to standard weight of 100 gm.

[2] Corrected to standard weight of 240 gm.

In the four comparisons provided by the two experiments, and the two weights of rats, the dynamic effect of the mixed supplement was in each case much lower than could have been computed from the individual values as determined for the protein, carbohydrate and fat of which the supplement was composed; and in three comparisons among the four the dynamic effect of the mixed supplement was either as low as or lower than the dynamic effect of the lard, which was in all cases lower than that of the carbohydrate or of the protein.

There is, therefore, a nutritive supplementation of the three main classes of nutrient compounds in combination which profoundly influences their dynamic effects.

In other words, the dynamic effects of individual nutrients are not significant with respect to combined nutrients, as in all nutritive practice, though popular impression is to the contrary. If such values are not to mislead they must be used in a critically guarded manner, for they have no general nutritive significance.

The net energy of the beef muscle protein is the lowest because of its low metabolizability and high heat increment; dextrin has a higher net energy value than has beef muscle protein, while the value for lard is much higher still.

As the evidence stands—the net energy values for the beef muscle protein and the dextrin were higher, and the value for the lard was lower, for the older than for the younger rats.

SUMMARY

The dynamic effects and net energy values of beef muscle protein, dextrin and lard were determined, separately and in combination, by feeding superimposed upon an approximately complete basal diet.

The subjects were growing, male, albino rats, and heat was measured by the open-circuit Haldane procedure.

The point of view of the experimenters is that even though a nutrient is fed alone it is never utilized alone, and that the

combination in which it is metabolized largely determines its dynamic and net energy values.

Among the individual supplements the dynamic effect of the beef muscle protein was the highest, that of dextrin was intermediate, and that of lard the lowest; while the dynamic effect of a mixture of the three nutrients was in all cases much lower than would be a value computed from the individual measurements for the three components. Dynamic effects as determined for individual nutrients, therefore, are not significant with reference to combined nutrients.

In seven instances among eight the dynamic effects were higher for the 100-gm. than for the 240-gm. rats.

The net energy of the beef muscle protein was the lowest because of its low metabolizability and high heat increment; dextrin has a higher net energy value than has beef muscle protein, while the value for lard is much higher still.

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THE VITAMIN D REQUIREMENT OF YOUNG TURKEYS

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TWO FIGURES

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INTRODUCTION

It was observed by Scott, Hughes and Loy ('32) that the vitamin D requirement of young turkeys was higher than that of chicks. Baird and Greene ('35) reported that chicks required 18 U.S.P. units of vitamin D per 100 gm. of diet and turkeys required 60 to 70 units. The experimental period lasted 12 weeks, and the data indicated that the requirement of turkeys for vitamin D expressed in units per 100 gm. of diet, was greater for the first 4 weeks of their lives than for the ensuing 8 weeks. Baird and Greene found that turkeys receiving 0.35% of fortified cod liver oil in the diet had an average tibial ash content of 43.9% at 4 weeks. Higher levels of the oil, which contained 250 units (U.S.P. X, revised 1934) of vitamin D per gram, were not fed.

Recently the A.O.A.C. chick unit (Association of Official Agricultural Chemists, 'Methods of Analysis,' 1935, p. 351) has come into wide use in testing sources of vitamin D used in poultry feeding. The present investigation was initiated with the intention of finding the vitamin D requirement of young turkeys in terms of A.O.A.C. chick units.

EXPERIMENTAL

A. Experiments with turkeys

In a preliminary experiment, a group of turkeys was kept for 4 weeks on a diet to which no vitamin D was added. At the end of the experimental period the average tibial ash content was found to be 29.2%. A second group received a supplement of 0.3 gm. of a fish oil blend stated to supply 400 A.O.A.C. chick units of vitamin D per gram, to 100 gm. of diet. The tibial ash content of this group was 48.0%. It seemed from these results that Baird and Greene ('35) might not have reached the level of vitamin D necessary for maximal calcification.

In the next experiment, the following basal diet was used for turkeys (diet PD): ground yellow corn, 25%; ground barley, 25; ground wheat 7; wheat bran, 15; sardine meal, fat extracted, 17.5; dried skim milk, 5; dehydrated alfalfa meal, 7.5; ground limestone, 1.5; steamed bonemeal, 1; and salt, 0.5. It contained approximately 23% of protein, 2.0% of calcium, and 1.0% of phosphorus. Except for the omission of the vitamin D supplement, the diet was a typical turkey starting diet.

Turkeys were hatched from eggs laid by the flock at the Poultry Division, Davis. The parent birds were kept in outside yards and were not housed; their diet consisted of approximately equal parts of whole grains and a mash containing 0.5% of sardine oil. The sardine oil was stated to contain a minimum of 100 A.O.A.C. chick units of vitamin D per gram. Undoubtedly the parent birds received a large additional supply of vitamin D from direct sunlight. Turkey poults were removed from the incubator on the day after hatching and fed the experimental diets immediately. The birds were kept in electrically heated, battery brooders with wire floors in a room from which daylight was excluded. From eleven to twenty birds were used in a group. Several varieties and crosses were used with the Bronze variety predominating. The birds were weighed at hatching and weekly thereafter.

They were killed on the twenty-eighth day, and the tibiae were removed, cleaned and placed in 95% ethanol. The bones were then extracted in a Soxhlet apparatus for 24 hours with anhydrous isopropanol; then they were turned end for end in the extractors and the extraction continued for a further 24 hours. Following this, the bones were extracted with isopropyl ether for 30 hours and were similarly reversed halfway through the ether extraction. The extracted bones were placed individually in tared Sillimanite crucibles, dried at 110° for 24 hours and weighed. Drying was repeated for 36 hours, following which the crucibles were reweighed. The crucibles were then placed in a cold electric muffle furnace, the temperature of which was raised to 750° during a period of 1 hour. This temperature was maintained for 2 hours, and the weights of the residues from the combustion were used to calculate the percentage of ash.

Sources of vitamin D were diluted with cottonseed oil [1] and mixed with the diets so that oil was added to all diets at the rate of 2 gm. to 98 gm. of diet. Reference cod liver oil, control 1-38-D containing 95 U.S.P. XI vitamin D units in 1 gm. of the oil, was purchased from the Board of Trustees of the United States Pharmacopeial Convention. The A.O.A.C. chick unit, by definition, is the potency of one unit of U.S.P. reference cod liver oil measured with chicks. Hence the use of this oil should enable a direct determination of the vitamin D requirement of turkeys in terms of A.O.A.C. chick units. The other oil used in the investigation was a fish oil blend [2] consisting of sardine oil fortified in vitamins A and D from the untreated liver oils of various fish to a potency of approximately 400 A.O.A.C. chick units of vitamin D and 3000 U.S.P. units of vitamin A per gram. It will be referred to as 'fish oil 1.'

In the first experiment eight groups of poults were used. Group 1 received basal diet PD without supplementation with

[1] 'Wesson oil.'

[2] 'Sardilene 400.'

vitamin D. The other groups received supplements as shown in table 1.

It was expected that the diet received by group 6 would provide sufficient vitamin D for maximal calcification, and when the experiment was planned, groups 7 and 8 were included merely as 'positive control' groups. However, the results made it evident that 100 units of vitamin D added in the form of reference cod liver oil per 100 gm. of diet were far from sufficient for maximal growth and calcification. Three-tenths per cent of fish oil 1 was also insufficient, since growth and calcification were significantly greater when 0.5% was used. In figure 1 the calculated straight line of best fit has been plotted for the bone ash of groups 1 to 6 by use of the method of least squares. When this line is extrapolated, a value of 46.8% bone ash, corresponding to group 7, gives a vitamin D value of 189 'units' per 100 gm. of diet, corresponding to 630 'units' per gram of fish oil 1. The 'unit' obtained by this extrapolation is based on a comparison of U.S.P. reference cod liver oil with a source of vitamin D of unknown potency using the turkey as a test animal. Such a 'unit' may be referred to for convenience as a 'turkey unit,' and is an expression of the response obtained in turkeys from 1 U.S.P. unit of reference cod liver oil. The straight line extrapolation is not necessarily justifiable, since Massengale and Bills ('36) demonstrated that the response curve for chicks had a progressively diminishing slope and varied in shape with the source of vitamin D. If the response curve for turkeys diminished in slope in the region beyond the points corresponding to groups 1 to 6, an even higher value would be obtained for the vitamin D content of diet 7.

Curve A, figure 1, indicates that unit increase in calcification in chicks in the experiments of Massengale and Bills ('36) was produced by one-tenth of the increase in vitamin D unitage required to produce unit increase of calcification in turkeys in the present investigation. This statement applies only to a range of values in the lower and linear region of the response curve.

TABLE 1

Summarized results of first experiment with turkeys, hatched May 10, 1938 showing the relation of body weight, tibial weight and tibial ash to level of vitamin D in the diet. The birds were sacrificed at an age of 4 weeks

Group	*Supplement to 100 gm. of basal diet PD*			*Number of birds at 4 weeks*		*Average body weight at 4 weeks*			*Average weight of dry fat-free left tibiae*			*Average ash of tibiae*		
				Males	Females	Males	Females	Average of both sexes	Males	Females	Average of both sexes	Males	Females	Average of both sexes
	gm.	Oil	Units of vitamin D			gm.	gm.	gm.	gm.	gm.	gm.	%	%	%
1	None		0	7	9	186	173	180	0.592	0.508	0.550	28.27	31.22	29.74
2	0.21	CLO[1]	20	6	8	210	198	204	0.600	0.583	0.592	32.25	31.76	32.00
3	0.42	CLO	40	4	11	242	244	243	0.676	0.673	0.674	31.62	34.96	33.29
4	0.63	CLO	60	3	11	232	271	252	0.665	0.771	0.718	32.63	36.28	34.46
5	0.84	CLO	80	5	10	239	220	230	0.748	0.675	0.712	34.98	38.31	36.64
6	1.05	CLO	100	8	6	304	264	284	0.883	0.789	0.836	37.19	40.92	39.06
7	0.30	Fish oil 1		6	11	302	308	305	0.925	1.033	0.979	45.54	48.07	46.80
8	0.50	Fish oil 1		9	6	359	336	348	1.250	1.144	1.197	48.90	49.17	49.03

[1] CLO = U.S.P. reference cod liver oil.

In the second experiment, levels of 0.05, 0.1 and 0.2% of fish oil 1 were selected to be within the range covered by the levels of U.S.P. cod liver oil used, which were 0, 0.63 and 1.26% supplying respectively 0, 60 and 120 U.S.P. units of vitamin D per 100 gm. of diet. By this means, it was hoped to obtain a close evaluation of the potency of fish oil 1 as compared with reference cod liver oil using the turkey as a test animal.

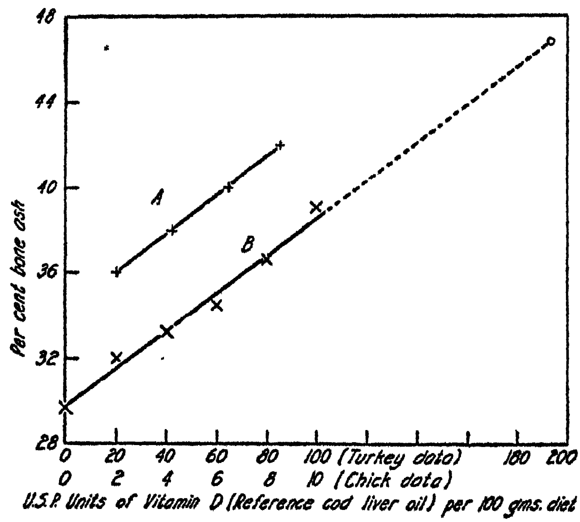

Fig. 1 Curve A, shown for comparison, was plotted from a short range of the data of Massengale and Bills ('36) for the femoral ash of chicks at 45 days of age, using a horizontal scale of one-tenth the value of the scale used for curve B. Curve B was plotted from the data of experiment 1 for the tibial ash of turkeys at 4 weeks of age. The points marked 'x' are the values obtained with U.S.P. reference cod liver oil. The calculated straight line of best fit was plotted for these points and was extrapolated as a dotted line to the point shown by a circle, which corresponds to the bone ash value of group 7, receiving 0.3 gm. of fish oil 1 per 100 gm. of diet.

Levels of 0.3 and 0.4% of fish oil 1 were included in the series in order to obtain further information on the level of this oil required for maximal growth and calcification. The results are shown in table 2. Growth was slower in groups 15 and 16 as compared with groups 7 and 8, probably because the poults in the second experiment were hatched later in the

TABLE 2

Summarized results of second experiment with turkeys, hatched June 21, 1938

Group	Supplement to 100 gm. of basal diet PD			Number of birds at 4 weeks		Average body weight at 4 weeks			Average weight of dry fat-free left tibiae			Average ash of tibiae		
				Males	Females	Males	Females	Average of both sexes	Males	Females	Average of both sexes	Males	Females	Average of both sexes
	gm.	Oil	Units of vitamin D			gm.	gm.	gm.	gm.	gm.	gm.	%	%	%
9	None		0	4	11	194	181	187	0.544	0.520	0.532	30.05	30.19	30.12
10	0.05	Fish oil 1		4	10	218	200	209	0.555	0.519	0.537	34.05	34.66	34.36
11	0.10	Fish oil 1		7	5	352	214	233	0.695	0.599	0.647	36.74	36.76	36.75
12	0.63	CLO [1]	60	5	7	281	225	253	0.811	0.619	0.715	37.92	38.04	37.98
13	0.20	Fish oil 1		8	5	267	235	251	0.819	0.727	0.773	42.31	44.86	43.58
14	1.26	CLO [1]	120	3	7	258	265	262	0.779	0.830	0.804	42.47	44.26	43.36
15	0.30	Fish oil 1		3	8	261	281	271	0.765	0.818	0.792	46.03	46.29	46.16
16	0.40	Fish oil 1		7	4	300	256	278	1.004	0.841	0.922	50.00	50.02	50.01

[1] CLO = U.S.P. reference cod liver oil.

season and hence grew more slowly (Asmundson and Lloyd, '36).

Acute symptoms of vitamin D deficiency were observed in turkeys on the basal diet. At 14 days of age the birds manifested a tendency to squat. After the twenty-first day, some of the birds were unable to stand. Four birds in group 1 died during the fourth week. No birds in any of the other groups, including group 9, died during the fourth week. Three birds in group 1 had bone ash values of less than 26%.

In order to evaluate the potency of fish oil 1, a calculated straight line of best fit was plotted for the bone ash values obtained from groups 9, 12 and 14 using the same coordinates as in figure 1. The bone ash percentages of groups 10, 11 and 13 when plotted on this line gave respective values of 34, 57 and 118 'turkey units' of vitamin D per 100 gm. of diet. These values correspond to 680, 570 and 590; mean 613 $\pm$ 48 'turkey units' of vitamin D per gram of fish oil 1. The first experiment had indicated roughly that fish oil 1 contained about 630 'turkey units' of vitamin D per gram. The values obtained in both experiments with turkeys indicated that fish oil 1 had an unexpectedly high vitamin D content, in view of the fact that the oil supposedly contained about 400 chick units per gram. It hence was thought desirable to test fish oil 1 with chicks in order to find the number of chick units of vitamin D per gram.

B. Evaluation of fish oil 1 with chicks

Single-comb White Leghorn chicks were used. In experiment 3 the chicks were hatched from eggs obtained from the University flock at Berkeley. The parent birds received a diet consisting of approximately equal parts of whole grains and a mash containing 0.3% of a fish oil stated to contain a minimum of 400 A.O.A.C. chick units of vitamin D per gram. The parent birds also had access to yards giving them contact with direct sunlight. The other experimental details were as described for turkeys. Diet PD was used as the basal chick diet. The results are summarized in table 3.

The comparatively high level of bone ash in group 17 receiving basal diet PD indicates that the diet did not produce acute rickets in chicks in contrast to the results with turkeys. The values obtained for body weight and tibial ash do not show the consistent trends obtained with turkeys. For evaluation of fish oil 1, the same procedure was used as in experiment 2, a straight line of best fit being plotted from the bone ash values obtained from groups 17, 18, 19 and 20. The bone ash percentages of groups 22, 23 and 24 when plotted on this line gave respective values of 6.1, 13.1 and 12.7 chick units of

TABLE 3

Comparison of vitamin D potency of U.S.P. reference cod liver oil with fish oil 1, using chicks as test animals and diet PD as basal diet

Group	*Supplement to 100 gm. of basal diet*			*Number of birds at 4 weeks*		*Average body weight at 4 weeks*	*Average ash of tibiae*
	gm.	Oil	Units of vitamin D	Males	Females	gm.	%
17	None		0	5	7	168	39.9
18	0.105	CLO[1]	10	7	5	178	42.8
19	0.157	CLO	15	4	7	165	44.9
20	0.210	CLO	20	8	4	184	44.5
21	0.262	CLO	25	5	6	181	46.1
22	0.0125	Fish oil 1		6	4	195	41.8
23	0.0250	Fish oil 1		6	6	167	43.5
24	0.0375	Fish oil 1		3	9	169	43.4
25	0.0500	Fish oil 1		6	6	178	46.3
26	0.1000	Fish oil 1		6	5	186	48.1

[1] CLO = U.S.P. reference cod liver oil.

vitamin D per 100 gm. of diet. These values correspond to 488, 524 and 339; mean 450 ± 80 units of vitamin D per gram of fish oil 1. Groups 21, 25 and 26 were omitted from the calculation because the bone ash values were in the upper and non-linear region of the response curve (Massengale and Bills, '36). The sex ratios of the groups in experiment 3 fell within the limits shown by Massengale and Bills ('36) to be inconsequential with respect to sex error of bone ash.

The results indicated that fish oil 1 contained fewer 'chick units' than 'turkey units' of vitamin D per gram when

measured by means of the same basal diet and reference cod liver oil. However, the results with chicks in experiment 3 showed a wide variation, and it was hence thought advisable to assay fish oil 1 by means of a modified A.O.A.C. method in the expectation of obtaining a more accurate evaluation of the potency of fish oil 1 in terms of 'chick units.' The following basal diet was used: ground yellow corn, 56%; wheat middlings, 25; casein, 10; dried skim milk, 5; dried yeast, 1; bone meal, 1; calcium carbonate, 1; sodium chloride, 1. Chicks from a commercial hatchery received the basal diet for 10

TABLE 4

Comparison of vitamin D potency of U.S.P. reference cod liver oil with fish oil 1 using chicks as test animals with a modified A.O.A.C. basal diet

Group	*Supplement to 100 gm. of basal diet*			*Number of birds at 31 days*	*Average body weight at 31 days*	*Average ash of tibiae*
	gm.	Oil	Units of vitamin D		gm.	%
27	None		0	12	195	33.9
28	0.052	CLO [1]	5	13	193	35.5
29	0.105	CLO	10	14	225	38.5
30	0.157	CLO	15	14	247	40.0
31	0.210	CLO	20	15	234	44.4
32	0.262	CLO	25	14	245	44.0
33	0.020	Fish oil 1		15	226	37.9
34	0.030	Fish oil 1		14	225	39.0
35	0.040	Fish oil 1		15	223	42.4

[1] CLO = U.S.P. reference cod liver oil.

days and then were fed the test diets for the next 21 days, after which they were killed and the bone ash content of the tibiae determined as described above. The results are summarized in table 4. For evaluation of fish oil 1, the same procedure was used as in experiment 3 except that the straight line of best fit was plotted from the bone ash values obtained from groups 27, 28, 29 and 30. The bone ash percentages of groups 33, 34 and 35 when plotted on this line gave respective values of 9.7, 12.2 and 20.2 chick units of vitamin D per 100 gm. of diet, corresponding to 485, 407 and 505; mean 466 $\pm$ 42 units of vitamin D per gram of fish oil 1. Hence

experiment 4 gave a value for fish oil 1 similar to that obtained in experiment 3.

DISCUSSION

It has been demonstrated by Bills, Massengale, Imboden and Hall ('37) and Bills, Massengale, Hickman and Gray ('38) that fish oils may contain varying mixtures of the several forms of vitamin D. A comparison of the efficiency of these various forms when made with rats is different from a comparison made with chicks. This fact led Bills, Massengale, Imboden and Hall ('37) to speak of the 'efficacy ratio' of a fish oil as a measure of the comparative response of rats and chicks to 1 unit of the given fish oil, the basis of standardization being in each case a reference cod liver oil which was assigned a value of 100. The efficacy ratio (perhaps a better term would be 'efficacy quotient') of a fish oil is an expression of the resultant of the efficacy ratios of the various forms of vitamin D contained in the oil. The fact that the efficacy ratio of a fish oil often departs far from the value of 100 has led to the widespread adoption of the chick as a test animal for sources of vitamin D which are to be used in feeding chickens. The present investigation indicates the possibility of the existence of a difference between chicks and turkeys in their response to various sources of vitamin D, and suggests that inaccuracies may be caused by use of the chick test in evaluating sources of vitamin D to be used in feeding turkeys. Fortunately the issue is not of great practical importance, since a margin of safety may be easily provided in rations fed to young turkeys, and older birds commonly receive most or all of their vitamin D requirement from direct sunlight.

The possibility remains that the difference in response observed between chicks and turkeys was due to differences in the amount of uptake from the gut. No evidence is available on this point, although Klein and Russell ('31) have shown that the low efficacy of irradiated ergosterol for chicks is not due to a poor uptake.

The efficacy ratio of a fish oil, determined by the rat: chick method, varies with the degree of calcification produced (Bills and co-workers, '37). This may also be the case when chicks and turkeys are compared, but the data in the present investigation are not sufficiently extensive to illustrate the point.

A straight line appeared to be the best form of curve to fit to the bone ash values obtained from groups 1 to 6 (fig. 1). This was not unexpected, since Massengale and Bills ('36) using chicks observed that 'beginning at a threshold corresponding to the first definite betterment of the ricketic bone,

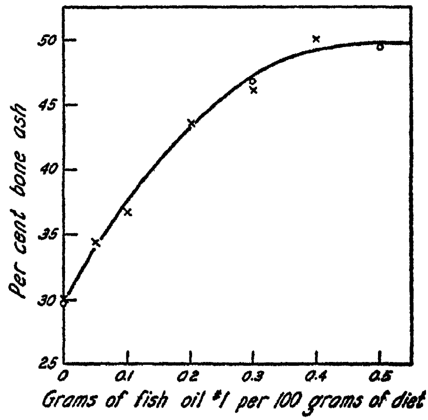

Fig. 2 Response curve obtained by plotting tibial ash values of turkeys at 4 weeks of age against levels of fish oil 1. Circles represent values obtained in experiment 1, crosses represent values obtained in experiment 2. One gram of fish oil 1 supplied about 620 'turkey units' of vitamin D when compared with U.S.P. reference cod liver oil, using the turkey as a test animal. The curve is the parabola of best fit, calculated by the method of least squares, and produced in a straight line beyond its point of inflexion (49.6% bone ash). The results indicate that maximal calcification necessitated levels of vitamin D in the neighborhood of 250 'turkey units' per 100 gm. of diet.

and continuing halfway toward normal bone, the relation of units to ash is essentially linear.'' A straight line was also used for the evaluation of fish oil 1 in the other experiments because the points used for evaluation fell in the linear region. When the effect of higher levels of vitamin D on turkeys was examined by means of fish oil 1, a parabola (fig. 2) seemed to correspond well to the observed data.

The turkey was found to be a sensitive test animal for the study of vitamin D. Bone ash values extended over a range of from 30 to 50% when a range of vitamin D levels was used which extended from 0 to over 200 units per 100 gm. of diet. Moreover, the diet was not distorted for the purpose of rachitogenesis; indeed, the bone ash value of 39.9% obtained from group 17 of chicks (table 4) on the turkey basal diet indicated that the diet failed to produce acute rickets in chicks, which further emphasizes the high vitamin D requirement of young turkeys. The favorable levels of calcium and phosphorus in the turkey basal diet may have accounted for the mild nature of the rickets in group 17 (compare Dols, '36).

An examination of the data in tables 1 and 2 shows that the average bone ash of the females was greater than that of the males in fifteen of the sixteen groups, the mean difference being 1.56 ± 0.38. This difference cannot be explained on the basis of slower growth leading to greater calcification because the average body weight of the females was actually greater than that of the males in five of the sixteen groups, and the average weight of the tibiae of the females was greater than that of the males in four groups. This sex difference in rate of calcification may have some relation to the observation of Marsden ('31) that male turkeys tended to develop deformed breast bones more readily than females.

The relation of growth of turkeys to the level of vitamin D fed was quite marked. In the first experiment the birds which received the highest level of vitamin D supplementation weighed 93% more than the birds on the basal diet at 4 weeks. Intervening levels of vitamin D produced growth responses which indicated some correlation between growth and calcification.

Massengale and Bills ('36) regard a bone ash of between 46 and 47% in chickens as normal under the conditions of their experiments. In the present communication, no attempt is made to define the 'normal' level of bone ash for turkeys, but it may be noted that in both of the experiments involving turkeys, the highest body weight at 4 weeks accompanied the

highest level of calcification, which was 49.03% in the first experiment and 50.01% in the second.

SUMMARY

1. An investigation of the vitamin D requirement of young turkeys was made by measurement of the ash content of the tibiae at 4 weeks of age as influenced by various levels of vitamin D in the diet.

2. Under the conditions of the experiment, the highest bone ash was in the neighborhood of 50%.

3. U.S.P. reference cod liver oil produced unsatisfactory growth and calcification when added at a level of 120 units (U.S.P. or A.O.A.C.) per 100 gm. of diet. Higher levels were not fed.

4. A linear relationship between vitamin D level and bone ash was observed in an experiment in which reference cod liver oil was added at levels ranging from 20 to 100 units per 100 gm. of diet.

5. Growth rate of turkeys was markedly influenced by vitamin D; in one experiment the birds receiving an ample supply of the vitamin weighed 93% more than the birds on the basal diet at 4 weeks.

6. Satisfactory growth and calcification in complete absence of sunlight were produced when turkeys received 0.4 gm. of a fish oil blend per 100 gm. of diet. Significantly poorer calcification was produced when 0.3 gm. was used. The oil was tested with chicks and was found to supply approximately 450 units of vitamin D per gram when the turkey basal diet was fed, and 466 units when a modified A.O.A.C. basal diet was used, based on a value of 95 units of vitamin D per gram for U.S.P. reference cod liver oil.

7. The fish oil blend functioned as a source of vitamin D that was more effective, chick unit for chick unit, than reference cod liver oil for turkey poults. The average turkey: chick efficacy ratio of the fish oil blend was about 140 (cod liver oil = 100). Hence the vitamin D requirement of turkeys could not be precisely stated in terms of A.O.A.C. chick units,

since the possibility of a differential species response between chicks and turkeys is not considered by such an expression. A value of 200 A.O.A.C. chick units of vitamin D per 100 gm. of diet may be taken as an approximation of the requirement of turkeys for the first 4 weeks to produce maximal calcification in the complete absence of sunlight.

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THE EFFECT OF APPLE CONSTITUENTS ON THE RETENTION BY GROWING RATS OF LEAD CONTAINED IN SPRAY RESIDUES [1]

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The value of any food material depends both upon the nutrients contained in it and their availability in covering animal requirements, and upon the physiological effect of the food. Under the latter heading is included the effects of the food material in modifying body processes: digestion, heat production, resistance to disease, etc. Most foods are evaluated entirely upon their content of available nutrients, but a few, although they may rate high according to nutrient content, are also of value in the diet because of some specific physiological effect on the body. Among such foods are apple, banana, wheat bran, lactose, gelatin and liver.

Although in many respects the apple is a good food on the basis of its nutrient content (Todhunter, '37), it is not preeminent with respect to any one nutrient. However, the apple has been found to possess many remarkably favorable properties quite aside from its value in covering the nutritive requirements of the body. Its efficacy in the prevention and treatment of diarrheal conditions and other intestinal disorders, particularly in infants, has been frequently demonstrated (Manville, Bradway and McMinis, '37; Manville, '38).

[1] This investigation was conducted under the supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

This favorable action has been ascribed with much reason to the pectins contained in apple (Baumann and Forschner-Böke, '34; Malyoth, '34); the finding that a commercial pectin is much less efficacious than apple powder (Frank, '37) may be a result of the difference in chemical and physical properties of commercial pectin and the pectin of apple powder (Norris and Resch, '37). Human subjects fed apple powder containing pectin 1 hour before receiving 200 cc. of milk inoculated with B. prodigiosus passed no viable bacteria of this type in the feces, while with the same subjects receiving inoculated milk but no pre-meal of apple powder, recoveries of 43 to 94% of viable B. prodigiosus in the feces were secured (Bergeim, Hanszen and Arnold, '36). In experiments on rabbits Sullivan and Manville ('38) showed that the addition of dehydrated apple to the diet definitely promoted the growth of acidophilic organisms in the intestines and raised the hydrogen-ion concentration at all levels of the intestinal tract. Besides these effects of apple constituents within the digestive tract, evidence has been reported that pectin contributes to the detoxication mechanism of the body (Manville, Bradway and McMinis, '36), apparently by providing galacturonic acid for conjugation, and that the oral administration of pectin diminishes the coagulation time of the blood (Riesser and Nagel, '35; Aragona, '36).

These desirable properties of the apple should commend it as a food in the human diet. Unfortunately it is necessary at the present time to market apples containing residues of acid lead arsenate used as an orchard spray in the control of insect pests. Although the permissible amounts of residual lead and arsenic on commercial apples are under the control of the Federal Food and Drug Administration in the case of interstate shipment and of state agencies in a number of the states, the situation is not entirely a satisfactory one, and many attempts have been and are being made to alarm the consumer and to discourage the buying of apples. Even discussions of the question by public health authorities (Hanzlik, '37) can

hardly fail to add to the disquieting effect of less responsible writers.

It occurred to the authors, on the basis of the known properties of the apple, that possibly the constituents of this fruit would adsorb the lead contained on its surface or in some way inhibit or impede its absorption from the intestinal tract and thus diminish the possibility of a harmful effect. It seemed likely that the pectic or hemicellulosic constituents might have this effect, since pectic acid solutions are precipitated by lead, calcium and barium. In fact, a quantitative method for the estimation of pectin by hydrolysis and precipitation as calcium pectate in acetic acid solution has been proposed (Carré and Haynes, '22), and lead salts are quite generally less soluble than the corresponding calcium salts.

PLAN OF EXPERIMENT

The plan of the experiment involved the feeding of twelve trios of weanling rats for the purpose of determining the lead retention on three experimental diets. The first diet was a synthetic one, planned to be complete and to equal the apple powder diets in calcium and phosphorus. The second diet contained 32% of unsprayed-apple powder, replacing agar and starch in diet 1. The third diet was like the second except that sprayed-apple powder was used in place of the unsprayed. The lead content of all three diets was the same, namely, 33 p.p.m., supplied almost entirely by the spray residue in diet 3 and by $PbHAsO_4$ in diets 1 and 2. A comparison of the lead retention promoted by diets 1 and 2 would indicate the effect of apple powder; a comparison of the retention of lead on diets 2 and 3 would indicate the relative assimilability of the lead in $PbHAsO_4$ and in the residue from lead arsenate sprays. The composition of these diets is shown in table 1.

The apple powder in diet 2 was prepared from unsprayed Huntsman apples, that in diet 3 from sprayed Jonathan apples. The whole apples including skin and core, were sliced in thin pieces and dried in a current of warm air not exceeding 70°C. in temperature. They were then placed in desiccators

for 48 hours over $CaCl_2$, transferred to stone jars in cold storage at —7°C. and ground to a powder at that temperature. The unsprayed-apple powder contained 6.28% crude fiber, 2.36% nitrogen, 2.45% ash, 0.077% calcium, and 0.84 p.p.m. of lead. The sprayed-apple powder was analyzed only for lead and was found to contain 96.4 p.p.m. These apples had purposely not been washed to remove the spray residue.

TABLE 1

Composition of the experimental diets

CONSTITUENTS	DIET 1 %	DIET 2 %	DIET 3 %
Apple powder	0	32.0 [2]	32.0 [3]
Dried extracted egg	21.5	21.5	21.5
Lard	8.5	8.5	8.5
Butter	5	5	5
Salts mixture [1]	11.5	11.5	11.5
Cod liver oil [4]	1	1	1
Wheat germ oil	0.5	0.5	0.5
Dried yeast	10	10	10
Sucrose	8	8	8
Starch	27.75	2	2
Agar	6.25	0	0
$PbHAsO_4$	54 p.p.m.	54 p.p.m.	0
Total	100	100	100
Lead	33 p.p.m.	33 p.p.m.	33 p.p.m.
Calcium	0.54%	0.51%	0.51%
Phosphorus	0.63%	0.66%	0.66%

[1] A modified Osborne and Mendel mixture, containing cobalt, zinc and copper and 76% of starch for convenience in handling. In this mixture, all of the calcium and phosphorus is in the form of $CaHPO_4$.

[2] Unsprayed-apple powder.

[3] Sprayed-apple powder (lead arsenate spray).

[4] Guaranteed to contain 3000 U.S.P. units of vitamin A and 400 U.S.P. units of vitamin D per gram.

One rat in each trio received diet 1, a second rat diet 2 and the third rat diet 3. These diets were fed in equal amounts daily within each trio but the weights of the rats in each trio were kept constant by feeding sugar in concentrated solution to the rats growing at the slower rates. The rats in each trio were matched for sex, body weight and litter

membership. They were confined individually in special cages made of tin-plated wire mesh and block tin sheets held together by lead-free solder, and particular care was taken that no contact with metallic lead was possible. With nine trios the experiment was terminated when 1 kg. of experimental diet had been consumed. Because of a temporary insufficiency of food supply, the feeding of three of the trios was terminted with the consumption of 723, 650 and 750 gm. of experimental diet, respectively.

At the end of the feeding periods, which varied in length from 89 to 173 days for the various trios, the rats were killed with ether, the body lengths measured from tip of nose to root of tail, the contents of the alimentary canal removed and the 'empty weight' determined. The carcasses were then washed to remove all possibility of lead contamination and ashed at a temperature of 500° C. preparatory to lead analysis, which was carried out according to the diphenylthiocarbazone method, using the colorimetric procedure as explained in a previous publication from this laboratory (Shields, Mitchell and Ruth, '39).

In order to determine the retention of lead by the experimental rats during the period of feeding it was necessary to sacrifice a number of check rats at the beginning of the experiment for lead analysis. Nine such rats taken from the same litters as the experimental rats and weighing approximately the same were used for this purpose.

THE RESULTS OF THE EXPERIMENT

The results of the experiment are summarized in table 2. It will be noted that results on only two rats are reported for trio 2. One of the rats, receiving diet 2, escaped from its cage before the termination of the experiment and was never recovered. The analyses of the nine check rats from which the initial lead content of the experimental rats was estimated revealed lead stores ranging from 0.026 mg. to 0.090 mg. and averaging 0.046 mg. Since there were no indications that the different litters represented in the experiment dif-

TABLE 2

The food consumption, growth data and lead analyses of the experimental rats

TRIO NUMBER	RAT NUMBER	DIETS FED	INITIAL WEIGHT	FINAL WEIGHT	GAIN	BODY LENGTH	FOOD CONSUMPTION	SUGAR CONSUMED	DAYS ON FEED	FOOD REFUSALS	INITIAL Pb CONTENT	FINAL Pb CONTENT	NET GAIN IN Pb
			gm.	*gm.*	*gm.*	*cm.*	*gm.*	*gm.*			*mg.*	*mg.*	*mg.*
1	393	1	46	223	177	22.5	723	19	91	4	0.046	0.768	0.722
	394	2	44	228	184	22.0	723	38.5	91	6	0.046	0.366	0.320
	395	3	43	232	189	22.7	723	15.5	91	2	0.046	0.500	0.454
2	396	1	37	170	133	21.0	1008	140	154	10	0.046	0.618	0.572
	398	3	40	181	141	20.1	1008	28.5	154	18	0.046	0.367	0.321
3	399	1	44	202	158	21.0	650	47	92	4	0.046	0.508	0.462
	400	2	38	204	166	20.6	650	24	92	8	0.046	0.350	0.304
	401	3	40	197	157	21.0	650	4	92	0	0.046	0.392	0.346
4	402	1	58	208	150	21.5	750	18	89	1	0.046	0.454	0.408
	403	2	58	208	150	21.6	750	29.5	89	2	0.046	0.367	0.321
	404	3	57	205	148	21.9	750	30.0	89	11	0.046	0.400	0.354
5	405	1	50	182	132	20.4	1000	35	159	9	0.046	0.506	0.460
	406	2	50	181	131	20.3	1000	9.5	159	8	0.046	0.430	0.384
	407	3	50	179	129	20.3	1000	44.5	159	23	0.046	0.326	0.280
6	408	1	42	175	133	19.8	1000	0	164	1	0.046	0.800	0.754
	409	2	42	170	128	20.6	1000	25.5	164	28	0.046	0.453	0.407
	410	3	42	170	128	20.0	1000	83.5	164	0	0.046	0.525	0.479
7	411	1	32	169	137	20.5	1000	0	173	4	0.046	0.710	0.664
	412	2	33	169	136	20.0	1000	30.5	173	13	0.046	0.367	0.321
	413	3	34	166	132	20.0	1000	75.0	173	16	0.046	0.396	0.350

8	414	1	34	162	128	20.3	1000	86.5	167	18	0.046	0.468	0.422
	415	2	34	160	126	20.5	1000	84.5	167	17	0.046	0.338	0.292
	416	3	35	162	127	20.0	1000	16.5	167	0	0.046	0.272	0.226
9	417	1	26	159	133	20.0	1000	86.0	157	1	0.046	0.586	0.540
	418	2	28	162	134	20.0	1000	45.0	157	20	0.046	0.382	0.336
	419	3	30	159	129	20.0	1000	47.5	157	16	0.046	0.358	0.312
10	420	1	28	224	196	22.7	1000	37.5	125	6	0.046	0.413	0.367
	421	2	26	192	166	22.1	1000	101	125	11	0.046	0.389	0.343
	422	3	27	232	205	23.0	1000	12	125	1	0.046	0.333	0.287
11	423	1	32	202	170	21.0	1000	115	143	8	0.046	0.628	0.582
	424	2	30	210	180	22.5	1000	75	143	8	0.046	0.534	0.488
	425	3	30	213	183	22.0	1000	6	143	4	0.046	0.310	0.264
12	426	1	30	169	139	20.3	1000	52	152	11	0.046	0.530	0.484
	427	2	30	174	144	20.5	1000	37	152	17	0.046	0.333	0.287
	428	3	30	176	146	20.0	1000	19	152	0	0.046	0.321	0.275
Averages													
Synthetic Diet (1)			38.3	187.0	148.8	20.9		53.0		6.4		0.582	0.536
Unsprayed A.P. Diet (2)			37.5	187.1	149.5	21.0		45.5		12.5		0.392	0.345
Sprayed A.P. Diet (3)			38.2	189.3	151.2	20.9		31.8		7.6		0.375	0.329

fered significantly in the amounts of accumulated lead (nor even that the heavier check rats contained more lead than the lighter), and since the check rats averaged practically the same in body weight as the experimental rats did initially, the initial lead content of all experimental rats was estimated at the same average value, 0.046 mg.

DISCUSSION

The growth of the rats within the various trios was equalized as far as body weight is concerned by adjusting the amounts of the sugar supplement given. However, since there were no consistent differences in the amounts of sugar required with the different diets, the numbers of food refusals or the attained body lengths, it is obvious that the three diets used were equally effective in promoting growth.

The lead contents of the rats at the termination of the experimental feeding, as well as the estimated amounts of lead stored during this period, reveal interesting and significant similarities as well as differences. The average lead retentions on the three diets were 0.536 mg., 0.345 mg. and 0.329 mg., respectively, equivalent to 1 to 1.5% of the lead ingested. The average difference in storage between diets 2 and 3 containing unsprayed-apple powder and sprayed-apple powder, respectively, was clearly insignificant statistically, using Student's method ('25) of analysis ($M = 0.0160$, $s = 0.0969$ and $P = 0.31$). With such a high value of P, the probability that random factors alone operated to produce the observed average difference in lead retention is far too high to be neglected. This comparison can be interpreted to mean that the lead contained in spray residue is just as readily absorbed and retained in the body as the lead in the original spray chemical, acid arsenate of lead, although weathering and leaching have undoubtedly hydrolyzed the arsenate to some extent (McDonnell and Graham, '17; Ruth and Kadow, '34); there is the possibility also of some combination of lead with the constituents of apple wax (Cohee and St. John, '34).

In parts of the country where only early summer sprays are used, a part of the lead may be in the form of lead sulfide because of the use of lime sulfur (calcium tetra and penta sulfide and thiosulfate), together with the lead arsenate, as a summer fungicide. The apples used in this experiment, however, had been sprayed with a full summer schedule of lead arsenate, so that the lead sulfide resulting from the use of lime sulfur early in the summer must have been a minor quantity, in comparison to the lead arsenate resulting from later sprays.

The average differences in lead storage between the rats on diets 1 and 2, and diets 1 and 3 were 0.191 mg. and 0.207 mg., respectively. In all trios the rat on diet 1 stored more lead than the rats on the apple-powder diets 2 and 3. The probabilities that fortuitous factors could have accounted for such consistent and marked differences are so small—less than 0.001 in both comparisons—that they may be neglected. It may be concluded, therefore, that the constituents of apples, either one or a combination of two or more, inhibit the assimilation of lead in the growing rat. In all probability this inhibition occurs within the alimentary canal and relates to absorption only. Under the conditions of this experiment the inhibition averaged 37% and for reasons stated in the introduction is probably traceable, wholly or mostly, to the pectic constituents.[2]

Thus, the apple contains within its tissues a substance or substances that exerts a considerable effect on intestinal absorbability of the lead component of the spray residue contained on its surface. Furthermore, in this experiment the ratio of lead to apple solids was 96 p.p.m. If the apple powder had been made from apples containing no more lead than the legal tolerance (0.025 grain per pound), the ratio of lead to apple solids would have been about 22 p.p.m. It seems quite likely that with this smaller ratio of lead to apple solids, the inhibiting effect of the latter on lead absorption would have been considerably greater than 37%.

[2] A solution of pectic acid, prepared from pectin by mild alkaline hydrolysis and acidified with acetic acid, will precipitate even small amounts of lead.

CONCLUSIONS

1. No evidence was obtained that the lead occurring in the spray residue on apples is any more or less, assimilable by the growing rat than the lead contained in $PbHAsO_4$, the original spray chemical.

2. There exists in the apple a substance or substances capable of depressing considerably the assimilability of lead. Under the conditions of this experiment the depression averaged 37%. Quite probably this depression in assimilability (retention in the body) relates to absorption from the intestinal tract only, and may be brought about by the pectic constituents of the apple. It seems quite likely that it would be greater with apples containing a smaller proportion of lead to solids than the apples used in this experiment, in which the proportion was more than four times that permissible in interstate commerce.

3. It would appear that feeding experiments concerned with the possible toxicity of lead in lead arsenate spray residues on fruits should involve the feeding of rations containing the fruit solids, unless it can be shown that the fruit itself does not modify the assimilability of the lead.

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THE ANTI-ACRODYNIC PROPERTIES OF CERTAIN FOODS [1]

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Since the anti-acrodynic vitamin (anti-dermatitis vitamin, vitamin B_6) was differentiated from the other components of the vitamin B complex (Gyorgy, '35) there has been a need for a survey of the anti-acrodynic potencies of various foodstuffs. Recently with the isolation of the crystalline vitamin B_6 by Lepkovsky ('38), Gyorgy ('38) and Kuhn and Wendt ('38 a), it has become apparent that in addition to vitamin B_6 another factor is needed by the rat for the cure of dermatitis. Gyorgy ('38) after determining the effect of the crystalline vitamin commented as follows: "Even the skin effect was not regularly attained unless a further supplement corresponding to the so-called 'filtrate factor' was added." Birch ('38) produced dermatitis in rats which were supplied with ample amounts of B_6 but which lacked this accessory factor. In this laboratory acrodynia has been only temporarily relieved by crystalline vitamin B_6;[2] a severe dermatitis persisted unless a 'filtrate factor' was fed as well. From this it is apparent that any assay of anti-acrodynic potency would be in error if interpreted solely as evidence of the amount of vitamin B_6 present. However, irrespective of the composite nature of the anti-acrodynic factor, information regarding its

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

[2] Dr. S. Lepkovsky kindly furnished us a sample of the crystalline vitamin.

distribution as determined by biological assay should be of value. In anticipation of a similar need, Wilson and Roy ('38) have recently published a survey of the anti-dermatitis factor in Indian foodstuffs.

EXPERIMENTAL

The technic of assay was the same as that outlined by Quackenbush, Platz and Steenbock ('38). Weanling rats, reared by mothers on a potato diet low in the anti-acrodynic factor, were transferred to a basal diet of the following composition: cerelose (glucose) 78, casein (alcohol extracted) 18, Wesson salts 4. Each rat was given in addition a daily supplement of 5 micrograms calciferol and 10 micrograms β-carotene in 1 drop of the liquid portion of hydrogenated coconut oil, plus 20 micrograms riboflavin and 10 micrograms thiamin hydrochloride in 1 drop of N/50 acetic acid. The only components of the B complex which were supplied by this diet (diet V) were riboflavin and thiamin. Acrodynia was produced in 4 to 5 weeks.

When the acrodynia had developed to such a degree that the paws were erythemic, swollen and denuded, and the lips were fissured, swollen and denuded the ration was supplemented with the material to be assayed. These supplements were fed in small glass dishes. Unless otherwise noted vegetables and fruits were fed fresh, and legumes, seeds and cereals were fed air dried. Other materials were fed fresh or dried as indicated in table 1.

When a curative effect became evident, the mouth symptoms were the first to clear up, the swelling and soreness being reduced gradually. The forepaws healed before the hind paws with a disappearance of the erythema and the swelling. New fur started to grow on the healed areas. A scaliness usually persisted on the paws and tail for several weeks after the disappearance of the other symptoms. The supplement was assumed to be curative if at the end of a 3-week period the lips, eyes and ears were normal in appearance, and the paws and tail were free from all symptoms except a slight scaliness.

TABLE 1

The anti-acrodynic potency of foods

	MINIMUM CURATIVE LEVEL (MG./DAY)	MAXIMUM FAILING LEVEL (MG./DAY)	UNITS PER 100 GM.	WILSON AND ROY VALUES (RECALC.)
Green leafy vegetables				
1. Lettuce	4000	1500	25	77
2. Spinach	1500	1000	66	53
Vegetables				
3. Tomato	4000	1500	25	42
4. Potato (dried)	500	300	200	
(calc. as fresh)	2500	1500	40	75
5. Carrot	4000	1500	25	
6. Beet (red)	7500	5000	13	
Fruits				
7. Banana	1500	500	66	
8. Orange	6000	5000	16	23
9. Apple	5000	4000	25	57
Legumes, seeds, cereals				
10. Alfalfa leaves	170	40	600	
11. Beans (navy)	250	20	400	
12. Peanuts	60	30	1660	
13. Soybeans	80	60	1250	
14. Cornmeal	250	200	400	
15. Whole wheat bread	250	200	400	
16. Oat meal	300	250	330	
17. Flaxseed	100	80	1000	
18. Rice polishings	200	150	500	
19. Wheat germ	80	60	1250	
Animal products				
20. Egg white (boiled, dried)	...	500	...	
21. Egg yolk (boiled, dried)	40	10	2500	
22. Whole milk	2500	...	40	
23. Skim milk	7000	5000	14	
24. Cheese (Cheddar)	400	300	250	
25. Beef muscle (raw, dried)	800	500	125	
26. Beef muscle (roasted, dried)	800	500	125	
27. Haddock (dried)	500	150	200	
28. Pork liver (dried)	200	80	500	
Fats (animal)				
29. Beef tallow	300	20	330	
30. Butterfat	500	200	200	
31. Chaulmoogra oil	...	40	...	
32. Cod liver oil	...	40	...	
33. Lard	40	...	2500	
Fats (plant)				
34. Coconut oil	200	100	500	
35. Coconut oil (hydrogenated)	...	200	...	
36. Linseed oil (comm.)	40	20	2500	
37. Linseed oil (crude)	40	20	2500	
38. Linseed oil (refined)	...	40	...	
39. Linseed oil (hydrogenated)	...	100	...	
40. Peanut oil (ether ext.)	20	10	5000	
41. Peanut oil (benzine ext.)	20	...	5000	
42. Peanut oil (crude)	40	20	2500	
43. Rice oil (comm.)	40	20	2500	
44. Rice oil (ether ext.)	20	...	5000	
45. Soybean oil (ether ext.)	10	...	10000	
46. Soybean oil (ether ext.)	15	...	7500	
47. Tung oil	...	40	...	
48. Cottonseed oil (comm.)	10	5	10000	
49. Corn oil (comm.)	5	3	20000	
50. Wheat germ oil (comm.)	4	2	25000	
51. Wheat germ oil (ether ext.)	7	...	15000	
Miscellaneous				
52. Yeast (dried)	250	200	400	506

If the amount of supplement proved to be insufficient to effect a cure, a larger amount was fed to another rat. If a supplement effected a cure rapidly, progressively smaller amounts were fed until failure resulted. For comparison of the relative potencies of the materials examined a unit of anti-acrodynic potency was defined as the minimal daily supplement which would cure an acrodynia of moderate severity in 3 weeks. Wilson and Roy ('38) in adopting a similar unit used 1 week as the period of assay.

DISCUSSION

Although in most cases both the minimal curative and the maximal failing doses are reported (table 1), assay data were obtained for many of the materials at levels above or below these limits. For purposes of comparison the assays of Wilson and Roy ('38), converted into our units, have been included for those materials of which we had corresponding data. In performing this conversion it was arbitrarily assumed that an animal cured in 1 week by a certain daily portion (Wilson and Roy unit) would be cured in 3 weeks by a daily dose of one-third of that portion. The agreement for those materials which could be compared in this manner is fairly satisfactory, certainly within the limits of errors of the assumed 'conversion.'

In the interpretation of results allowance must be made for the possible influence of members of the vitamin B complex other than those directly involved in the cure of the acrodynia. In the feeding of the supplements it was noted that apart from the healing of symptoms varied rates of growth were obtained. What effect these varied rates of growth had on the requirements for the anti-acrodynic factor cannot be estimated here. The data obtained, therefore, can be used for comparative purposes only.

It is apparent that cereals are richer in anti-dermatitis potency than fruits and vegetables. Meat and fish occupy an intermediate position. This generalization is also the conclusion of Wilson and Roy for Indian foodstuffs of similar

classification. However, these materials are relatively poor sources of the anti-dermatitis factors when compared with the potencies of certain fats. Thus soybean, cottonseed, corn and wheat germ oils are among the most potent substances assayed. Wheat germ oil has a potency approximately sixty times that of maize. It is interesting to note that yeast is not a rich source of the anti-acrodynic factor as it ranked with the cereals in potency and was easily exceeded by many of the fats. It is also interesting to note that lard, which is a common ingredient of many experimental rations is actually six times as potent as yeast.

Whether the potency of fats is to be attributed to an actual B_6 content cannot be answered at the moment although Kuhn and Wendt ('38 b) have reported that the B_6 molecule contains two alcoholic and one phenolic group which would make an ester form of the vitamin at least a possibility. However, Birch ('38) and Quackenbush, Platz and Steenbock ('38) have traced the potency of fats to the unsaturated fatty acid fractions. Salmon ('38) likewise obtained cures with certain fats and fatty acids. Birch suggested that the unsaturated fatty acid factor is necessary for a cure of dermatitis in addition to B_6 on the basis that where B_6 failed to cure alone, the addition of fat made a cure possible.

Whatever may be the mode of action of anti-acrodynic fats it is true that they constitute an extraordinarily potent source of anti-acrodynic activity. Therefore these fats must be included in any survey of the anti-acrodynic potency of foodstuffs.

The evaluation of these findings for human nutrition is impossible at the moment since the role of vitamin B_6 in human nutrition is as yet undemonstrated. Indeed, apart from the rat, the only other species for which vitamin B_6 has been indicated to be essential are the dog (Fouts, Helmer, Lepkovsky and Jukes, '38) and the pig (Chick, Macrae, Martin and Martin, '38).

SUMMARY

Fifty-two materials have been assayed for their anti-acrodynic activity using the rat as the experimental animal. Fruits and vegetables were found to be poor sources, fish and meat fair, seeds, legumes and cereals relatively rich and certain vegetable fats extremely potent sources of anti-acrodynic activity.

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VITAMIN B_1 CONTENT OF HUMAN MILK AS AFFECTED BY INGESTION OF THIAMIN CHLORIDE[1]

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Much interest attaches to the question of the control of the composition of human as well as of cow's milk. If by suitable feeding desirable changes can be brought about particularly in the vitamin content of milk obvious advantages would result. Most of the efforts made to increase the vitamin content of cow's milk by feeding the cows vitamin-rich feeds have met with somewhat disappointing results, either very little increase being produced as in the cases of the water soluble vitamins or only small transferrals of the excesses fed as in the cases of vitamins A and D. Physiological control of the levels of both vitamins B_1 and C has been suggested as explaining the limited response of the milk vitamin content to forced vitamin feeding. Gunderson and Steenbock ('32) advanced this suggestion to explain lack of vitamin B increase in cow's and goat's milk following an increase in this vitamin in the ration and Hunt and Krauss ('31) came to the same conclusion after similar experiments on cows. Hess, Light, Frey and Gross ('32) administered vitamin B-rich irradiated yeast to cows and obtained no effect on the vitamin B_1 of their milk.

Less work has been done on the effect of vitamin feeding on human milk. McCosh, Macy and Hunscher ('31) determined

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the vitamin B_1 content of the milk of three women before and during administration of 10 gm. dried yeast daily. These women were producing rather extraordinary amounts of milk, 1.5 to 3 liters daily. The yeast did not affect the volume of milk nor was there any clear indication of an increase in its vitamin B_1 content although some other fraction of the B complex seemed improved. Donelson and Macy ('34) also found that the administration of 10 gm. of dried yeast daily had no effect upon the vitamin B_1 content of the mixed milk of women producing large volumes of milk. The milks had rather uniformly 0.1 'Sherman' unit of vitamin B per cubic centimeter. The vitamin G (probably riboflavin) of the yeast milk was definitely increased from 0.2 to 0.3 Sherman unit per cubic centimeter. Neuweiler ('38) gave thiamin by intravenous injection and by mouth to three lactating women and produced temporary increases in the vitamin B_1 of their milk. He used the thiochrome method of assay and found in this way 5 to 13 μg. per 100 ml. in human milk and 21 to 57 μg. per 100 ml. in cow's milk.

Two young women, one in the early and the other in a later phase of lactation agreed to act as subjects for this experiment. Subject A made a weighed record of her food intake during the first month of the study and both she and subject B kept a careful diary in terms of measures of all food eaten throughout the experiment. The probable vitamin B_1 content of these food intakes was estimated using the best available compilations of assays (Williams and Spies, '38). The quantity of milk produced was estimated in the case of subject A by weighing the infant before and after each nursing but this record was not made regularly by subject B. Both infants were healthy and growing normally at all times and both mothers remained well and made no change in body weight.

Subject B had for several years been troubled with a mucous colitis and had consequently taken a smooth diet which was probably rather low in vitamin B_1. Subject A on the other hand was a student of nutrition and since she was particularly conscious of possible vitamin B_1 deficiencies in her diet had

taken pains to include therein whole cereals, wheat germ and yeast during both her pregnancy and lactation.

A sample of breast milk was obtained once a day by the subjects using a hand pump and these samples were brought at once to the laboratory where they were kept frozen solid until they were used.

The method of assay was that of rat growth after preliminary depletion. Autoclaved yeast was used as source of the B_2 complex. Crystalline thiamin chloride or the International clay adsorbate or both were used as standards fed parallel

TABLE 1

Vitamin B_1 of the diet and human milk production

SUBJECT	PERIOD	MONTH OF LACTATION	SUPPLEMENT THIAMIN CHLORIDE	VITAMIN B_1 IN THE DAILY DIET		VITAMIN B_1 IN THE MILK	TOTAL MILK PRODUCED	GAIN IN WEIGHT OF INFANT
			Mg. per day	Micrograms thiamin	Int. units	Micrograms per 100 ml.	Liters per day	Ounces per week
A	1	5th	None	1650	550	32	0.84	3.2
	2	6th	5	1150	385	..	0.54	4.8
	3	7th	10	1250	418	25	0.50	4.0
B	1	3rd	None	1050	350	11	0.60	4.0
	2	4th	5	1140	380	20	(estimated)	4.8
	3	5th	14.2	1060	355	25	(estimated)	8.0

with the milk to groups of litter mate rats. The period of assay was 28 days, and the breast milk samples were fed within a day or two of the date of collection. Raw and pasteurized market cow's milk was also fed in several cases as a further test of the reliability of the assays.

The periods used are indicated in table 1. The decreasing milk production recorded for subject A was due to the gradual weaning of the infant. No actual measurement of milk volume was made for subject B, but the amount obtained by the infant was apparently adequate since the weight increments were satisfactory and no supplements were necessary.

After the first month in each case supplements of 5 mg. daily crystalline thiamin chloride [2] were ingested by the subjects and a new assay of the milk carried out. A third period of 1 month followed during which still larger supplements of the

TABLE 2

The assay for vitamin B_1 of human milk as affected by thiamin chloride intake

ASSAY	SUBJECT	DAILY SUPPLEMENT OF THIAMIN CHLORIDE	AMOUNT OF MILK FED PER RAT PER DAY	STANDARD GROUP	NUMBER OF RATS	GAIN IN 4 WEEKS	VITAMIN B_1 PER 100 GM.	
		mg.	*ml.*			*gm.*	*µg.*	*I.U.*[1]
1	A	None	7.7		4	28	32	13
Dec. 22, 1937 to				Thiamin chloride, 2.2 µg.	8	25		
Jan. 19, 1938				2.5 µg.	8	28		
				Intern. stand., 10 mg.	9	27		
			8.5	Cow's milk, raw	7	26	27	11
2	A	5	6.0		4	4		
Feb. 5 to Mar. 5, 1938	B	None	10.0		4	13	12	5
				Intern. stand., 10 mg.	6			
			8.4	Cow's milk, pasteurized	4	19	20	9
3	A	10	8.4		7	26	25	11
Feb. 25, to Mar. 25, 1938				Thiamin chloride, 3.0 µg.	5	38		
			8.4	Cow's milk, pasteurized	4	35	32	13
	B	5	8.5		8	22	20	9
				Yeast, 80 mg.	4	52	5000	2160
4 Apr. 23 to May 21, 1938	B	14.2	8.5		8	19	25	11
				Intern. stand., 10 mg.	4	20		

[1] In assays 1 and 3 both international standard and pure thiamin chloride were used as standards of comparison. In assays 2 and 4, only the international standard was used but the conversion factor was taken as 2.3 instead of 3 because this was the ratio found in the other two assays.

[2] The thiamin chloride under the name of Betaxin was supplied through the generosity of the Medical Research Department of the Winthrop Chemical Company.

thiamin chloride were taken. The details of the biological assays are given in table 2.

It will be noted that the milk produced by subject A during the first period was of high B_1 potency, slightly better than that of the raw cow's milk fed during the same assay. This was evidently a peak period of production for this subject.

Typical menus for 2 days taken by this subject during the first period are given here because of the interest attached to the food intake on which such a high vitamin B_1-containing milk was produced:

FIRST DAY:		SECOND DAY:	
Breakfast	*oz.*	*Breakfast*	*oz.*
Grapefruit	2⅞	Orange juice	4¼
Skim milk	9¼	Skim milk	7½
Whole milk	2½	Top milk	2¼
Wheat germ cereal	3¼	Whole wheat bread	1¾
Raisin bread	1½	Wheat germ cereal	1½
Sugar	⅛	Jam	¾
Evening lunch	*oz.*	*Evening lunch*	*oz.*
Milk	16	Skim milk	9
Egg	2	Cookie	½
Orange	2⅛	Dates	1
Whole wheat bread	1¼	Candy	1
Peanut butter	1		
Sugar	⅛		
Luncheon	*oz.*	*Luncheon*	*oz.*
Cheese	¾	Egg	2¼
String beans	1½	Orange	2¼
Lettuce	1	Skim milk	9
Apple	2¾	Cottage cheese	1
Whole milk	9	Wheat bread	1¼
Biscuit	1	Apple sauce	2
Cake	1	Peanut butter	¼
		Cookie	1
Dinner	*oz.*	*Dinner*	*oz.*
Shrimp salad	6	Potato	3¾
Cauliflower	2	Beef	3
Baked beans	2	Squash	2
Squash	2	Canned cherries	4
Apple sauce	3	Rutabaga	2
Skim milk	6	Lettuce	1½
Cake	2	Raisin bread	1
		Jam	¼
		Mayonnaise	⅛

In her second period while she was taking 5 mg. thiamin chloride daily, subject A voluntarily reduced her dietary intake of vitamin B_1 since it was assumed that the large supplement would make up for such reduction. But the vitamin B_1 of the milk produced was apparently reduced since daily doses of 6.0 ml. did little more than maintain the weight of depleted rats whereas in the former period 7.7 ml. doses produced as good gains as 10 mg. of the International clay adsorbate.

In the third period with a daily supplement of 10 mg. thiamin chloride subject A produced milk nearly as good as in the first period. For this subject it is obvious that the thiamin supplement offered no advantage. Possibly the maximum concentration of vitamin B_1 had been attained previous to the supplementation.

Subject B on the other hand produced a milk of low vitamin B_1 value during the first period, 11 μg. per 100 gm. This is in the range reported by Neuweiler ('38). Supplements of 5 and then of 14.2 mg. of crystalline thiamin chloride produced increases to 20 and 25 μg. per 100 gm. This subject reported that during the periods of supplementation the constipation from which she and the baby had suffered as a result perhaps of continued use of a non-residue diet, was immediately relieved. The amount of increase in the vitamin B_1 secreted in the milk was sharply limited. If the volume of milk produced daily be taken as 600 gm. in the second period 0.12 mg. of vitamin B_1 was secreted into the milk out of 5 mg. increased intake, and in the third period 0.15 mg. was similarly secreted out of 14.2 mg. increased intake.

The cow's milk values found in these assays, 27, 20 and 32 μg. per 100 gm., are in the range usually reported. No difference between the vitamin B_1 potencies of raw and pasteurized market milk can be deduced from these few observations.

DISCUSSION OF FOOD INTAKE AND MILK PRODUCTION[3]

The typical diet of subject A during the first period contained 1650 μg. of vitamin B_1, yielded 2230 calories and 90 gm.

[3] Our thanks are due to Dr. George R. Cowgill for suggestions on the preparation of this manuscript.

of protein, 60% of which was from milk, egg or meat. There were 90 gm. of fat yielding 810 calories and 265 gm. of carbohydrate yielding 1060 calories. Her estimated calorie requirement not including milk production was 1800 calories. Since her average daily milk production was 840 ml. with probable value of 500 calories the transformation of food into milk calories appears to have been accomplished with but little energy cost.

The total food taken by this subject is small in amount compared with the intakes recorded by Shukers, Macy, Donelson, Nims and Hunscher ('31) for their three subjects, but the latter were producing extraordinary daily amounts of milk, 1420, 2366 and 3134 ml. respectively, on intakes of 3800, 4500 and 4200 calories. The caloric intake and milk production of our subjects represent probably a more nearly normal performance for women supporting only one infant.

The total non-fat calories metabolized by subject A was 1420 at an estimated cost of 426 μg. of vitamin B_1 and 269 μg. of vitamin B_1 appeared in the milk. A positive balance of 955 μg. remains. If however the cost of transference of B_1 to milk be treble that of maintenance as has often been suggested from observation on rats, the margin is reduced to 537 μg., and if the subject's own maintenance requirement be similarly trebled because of lactation demands, the margin is wiped out and a deficit left.

It seems reasonable to assume however that this subject was in vitamin B_1 equilibrium during this period and that her maintenance need for 400 to 500 μg. and her production in milk of 200 to 300 μg. were met by an intake of approximately two and one-half times this amount of the vitamin.

In the third period A produced only 300 calories of milk daily and transferred to it only 125 μg. of vitamin B_1 in spite of the fact that she was receiving 10 mg. of thiamin chloride as well as 1250 μg. in her diet. If her maintenance need was the same as in period I and the same ratio of transformation held, at least 125 μg. of the crystalline synthetic thiamin chloride must have been utilized.

Less exact data as to food intake and milk output are available for subject B. However her diet was considerably richer in carbohydrate than that of subject A, with probably increased maintenance requirement of vitamin B_1. If this be taken as 500 μg., with milk content of 66 μg. her transformation efficiency in period I was better than that of subject A or else she was in negative vitamin balance. By the same type of calculation it might be deduced that in periods 2 and 3 when she received daily 5 and 14.2 mg. thiamin chloride and produced 120 and 150 μg. of vitamin B_1 in her milk little or none of the added thiamin chloride was utilized for the extra milk vitamin. The actual increase in milk vitamin produced was only about 54 and 84 μg. daily.

Since the infants in both cases grew and maintained normal appetites and were at all times in excellent health it is probable that they were receiving sufficient vitamin B_1. They received only the usual supplements of orange juice and cod liver oil, except that in periods 2 and 3 A's baby was given also small amounts of cooked cereal. If total non-fat calories of human milk be estimated at 34 per 100 ml. and if the thiamin (in micrograms) to non-fat calories ratio of Williams and Spies ('38) be used, that is 0.3, no danger of beri beri occurs when the milk carries 10 μg. of thiamin per 100 ml. According to this criterion subject B in period 1 produced a milk barely adequate to prevent beri beri in her infant. The immediate improvement in appetite and intestinal regularity exhibited by this infant during the following periods of supplementation is contributory evidence to the plausibility of this suspicion.

It may be concluded that an intelligent selection of ordinary foods such as that made by A in period I will insure an intake of vitamin B_1 ample for the needs of lactation without fortification or supplementation. Likewise a somewhat restricted diet such as that taken by subject B may bring about low vitamin B_1 content of the breast milk. In the former case large doses of thiamin chloride hampered rather than improved the excretion of B_1 into the milk, and in the latter case the increase in the milk vitamin was not great and probably

no more than could be obtained by increased efficiency in the use of the vitamin of the food. Perhaps the crystalline thiamin chloride when given in such large excess is mostly wasted through non-absorption as found by Schultz, Light and Frey ('38). They were able to detect only about 1 mg. in the urine when 5 mg. thiamin chloride were given daily and about 2 mg. when 10 mg. were given. The rest was found in the feces.

Our subject A appeared however to excrete less vitamin into the milk when these large supplements were given than when natural food mixtures only were used. Perhaps the concentrated thiamin in the intestine had an adverse effect upon absorption of food thiamin.

SUMMARY

Two healthy young women taking diets of widely different vitamin B_1 content were found to secrete milk of similarly different vitamin B_1 potency, 32 and 11 μg. per 100 gm.

When each of these subjects took 5 mg. of crystalline thiamin chloride daily during a period of 1 month no increase occurred in the vitamin B_1 of the milk of the higher potency but some rise was seen in that of lower potency. The new level of the latter milk was 20 μg. per 100 gm.

The first subject then took 10 mg. and the second 14.2 mg. thiamin chloride for a month. The new levels were 25 and 25 μg. per 100 gm.

Three assays of market samples of cow's milk showed 27, 30 and 32 μg. per 100 gm.

It is concluded that the level of vitamin B_1 in human milk is controlled in the lower brackets by the vitamin B_1 content of the diet but that as in cow's milk a maximum level exists above which the vitamin content cannot be raised even by massive doses of thiamin chloride. This maximum level appears to be the same in human and cow's milk, 25 to 32 μg. per 100 gm. of milk.

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THE COMPARATIVE TOXICITY OF FLUORINE IN CALCIUM FLUORIDE AND IN CRYOLITE [1, 2]

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The first toxic effect that appears in animals receiving fluorine compounds in water or food, and the one that persists longest as decreasing doses of fluorine are given, is a hypoplasia of the dental enamel known as 'mottled enamel' in its human manifestation. An experimental and histologic study of this condition in rats by Schour and Smith ('35), revealed that fluorine has a direct disturbing effect on the enamel epithelium and later a more severe effect on the formation of the organic matrix. Neither of these effects is the same as those of other dental disturbances of nutritional (vitamin A deficiency) or of endocrine (parathyroidectomy, hypoparathyroidism, hypophysectomy) origin.

The concentrations of fluorine in municipal water supplies that constitute public health hazards, solely with reference to the incidence of mottled enamel however, have been well defined. For public health purposes, Dean and Elvove ('35) have defined the minimal threshold of fluoride concentration in a domestic water supply as "the highest concentration of fluoride incapable of producing a definite degree of mottled enamel in as much as 10% of the group examined." They

[1] This experiment was made possible by the donation of funds to the University of Illinois by the Aluminum Company of America.

[2] This investigation was conducted under the supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

present evidence indicating that this critical concentration, determined on samples collected over a 12-month period, is approximately 1 part per million or slightly less, and this seems to be the concensus of competent opinion. Thus, the toxicity of fluorine in drinking water has been quite definitely determined, insofar as toxicity can be expressed in terms of concentration. While it must be true that the amount of fluorine-bearing water consumed is an important factor in the production of toxic effects, perhaps the all-important factor, the public health evaluation of a specific water supply must of course be based upon its content of potentially dangerous elements.

The use of fluorine compounds, such as cryolite (Na_3AlF_6) and barium fluosilicate in spraying fruits and vegetables for the purpose of insect control, represents another type of health hazard insofar as these compounds remain on the surface of the marketed food and are consumed. In recognition of this fact, the secretary of agriculture has imposed a legal tolerance on such spray residues of 0.02 grain of fluorine per pound of food, equivalent to about 2.9 p.p.m. This tolerance applies only in interstate commerce.

Since the toxicity of fluorine in water has been so clearly determined, within the limits of accuracy imposed by the only practical method of expression, it would seem feasible to estimate the toxicity of fluorine spray residues by determining, first, the relative toxicity of the fluorine compounds occurring in water and those used in fruit and vegetable sprays, and second, the relative toxicity of fluorine in the same chemical combination but consumed in water and in food. The present paper will be concerned with the first of these problems as it relates to cryolite, while the following paper will be concerned with the second.

In comparing the toxicity of fluorine as cryolite with that of fluorine in potable waters, it was assumed that the latter, insofar as it is in a non-ionized condition, is in the form of calcium fluoride, because 1) many of the naturally-occurring fluoride-bearing rocks, which would be the ultimate source

of the water-borne fluorine, contain their fluorine in combination with calcium, 2) calcium fluoride is the least soluble fluoride commonly found in nature, and 3) the solubility of calcium fluoride would permit concentrations of the element at 18° C. of about 8 p.p.m., and very few potable waters in this country contain more fluorine than this. Cryolite is many times more soluble than calcium fluoride, the relationship being 15 to 1 according to Carter ('28) and 23 to 1 according to Roholm ('37).

The solubility of inorganic salts does not necessarily determine their absorbability from the mammalian intestinal tract, except for compounds so slightly soluble that they are suitable for gravimetric chemical analysis. Furthermore, the differences in absorbability that are dependent upon differences in solubility would be expected to diminish as the amounts of the compounds consumed diminish. Confirming this expectation, De Eds and Thomas ('34) found that wide differences in the solubility of fluorine compounds did not occasion appreciable differences in the minimum toxic concentrations for rats. Smith and Leverton ('34) have come to essentially the same conclusion, namely, that at a concentration of 14 p.p.m., fluorine, regardless of the solubility of the compound used, gives the first outwardly discernible effect upon the incisor teeth of the rat that was regularly observed. However, there were indications in the latter experiment that, even at this low concentration, fluorine was more toxic in the form of sodium fluoride and barium fluosilicate than in the form of calcium fluoride and cryolite.

Obviously only these low levels of fluorine intake are of significance to the problem of the toxicity of fluorine in drinking water and in spray residues. Furthermore, since only small differences in toxicity between different fluorine compounds are to be expected at low levels of feeding, the compounds to be compared should be fed in water solution to avoid complications due to particle size. Also the consumption of both food and fluorine-bearing water should be equalized between comparative animals so that equal dosages of fluorine

will be consumed as well as equal amounts of food, since it is conceivable that a variable intake of food would modify the physiological effect of a given dose of fluorine. The experiment to be reported in this paper was planned according to these conceptions. Young albino rats served as subjects of the experiment, with the assurance that the data obtained as far as the comparative toxicities of CaF_2 and Na_3AlF_6 are concerned, are applicable to humans, since dental fluorosis in the rat and in the human are quite similar (Schour and Smith, '35).

PLAN OF THE EXPERIMENT

Twenty-four albino rats, averaging initially 57 gm. in weight, were divided into twelve pairs on the basis of sex, body weight and litter membership. They were fed individually by the paired-feeding technic on a ration of Purina Fox chow of the following percentage composition: dry substance 90.98, ether extract 5.68, crude fiber 3.48, nitrogen 3.96, ash 7.65, calcium 1.24 and phosphorus 0.86. The heat of combustion was 4.166 calories per gram and the fluorine content 3.04 p.p.m.

The cryolite used in this experiment was a synthetic product marketed as an insecticide. It contained 43.7% fluorine and about 15% of non-cryolite material, including hydrated oxide of aluminum and compounds of sodium, calcium and silicon. The calcium fluoride was a chemically pure product.

Each pair of rats received the same amount of food daily and the same amount of fluorine in the drinking water. The odd-numbered rats received water containing CaF_2 and the even-numbered rats water containing cryolite. In order to avoid any possibility of the solid food depressing or obscuring the toxic effects of the test substances administered in the water, the consumption of the food and of the water was separated as much in time as possible. The rats were allowed to eat from 8 to 9.30 A.M. and during this time the water consumption was restricted to the 4 to 5 cc. of distilled water that was mixed with the food to prevent scattering. From

9.30 A.M. to 6 P.M. the rats had access to neither food or water. At 6 P.M. they were given measured portions of the fluorine-containing water and after these were consumed they were given distilled water ad libitum until the following morning at 8 A.M.

To permit an estimate of the initial fluorine content of the experimental rats, six rats of approximately the same weight range as the experimental rats were analyzed after removal of the contents of the alimentary canal. The entire carcasses of these rats were analyzed for fluorine by the technic used with the soft-tissue samples of the experimental rats.

After approximately 14 weeks of feeding, the rats were killed with ether and the digestive tracts removed, emptied, washed with distilled water and replaced. After determining the empty weights, the carcasses were autoclaved at 12 pounds steam pressure for 30 to 45 minutes in order to facilitate the separation of the skeleton from the soft tissues. The latter were combined with all condensed water, finely ground and thoroughly mixed by hand. The bones, after removal of the teeth, were dried for 12 hours at 110°C., broken up and extracted with ether for 48 hours in a Soxhlet extractor. The extracted bone was then dried at 110°C. for 20 minutes, weighed and finely ground in a hand mill. The teeth, after removal from the carcass, were washed first in water to remove all adhering tissue, and then in ether, after which they were dried at 110° for 24 hours and weighed.

The soft tissue, bones and teeth were analyzed separately for fluorine. Samples of soft tissue were weighed out, mixed with 15 cc. of a 10% solution of magnesium acetate and ashed for 12 to 16 hours at temperatures of 500° to 550°C. in an electric muffle furnace. Samples of bone were ashed for 16 to 20 hours at 550°C., while the entire weight of teeth was ashed for 20 hours at 550° to 600°C., carefully ground in an agate mortar and re-ashed for 24 hours at the same temperature.

The fluorine was isolated from the ash of all samples by distillation as hydrofluosilicic acid according to the well-known

method of Willard and Winter ('33), and a duplicate distillation was carried out to remove completely all phosphates, in accordance with the recommendations of Churchill, Bridges and Rowley ('37). The first distillation was made with sulfuric acid and the second with perchloric acid. The distillation temperature was maintained at 140°C. by the introduction of steam at the proper rate into the distillation flask. The distillation was continued until 250 cc. of liquid had been collected. In the analysis of soft-tissue ash the chloride ion was removed from the first distillate by precipitation with $AgClo_4$, as suggested by Armstrong ('36).

The second distillates were made alkaline with a dilute solution of NaOH and were then evaporated on an electric hot plate at a temperature just below boiling. In the case of the bone samples, the Rowley and Churchill ('37) procedure was followed, employing an aqueous medium for the titration of the fluorine with thorium nitrate. In the case of the soft tissue and tooth samples, the micro method of Armstrong ('36) was used, which permits the determination of as little as 10 micrograms of fluorine in 10 cc. of distillate. The only departure from Armstrong's directions was the use of a stronger thorium nitrate solution, 0.0006 to 0.0007 N, instead of 0.0004 N.

A number of recovery tests were undertaken with the analytical procedures above described. In five tests with bone samples, to which fluorine was added in amounts equal to those expected in the samples themselves, the recoveries ranged from 96 to 101%, averaging 98.5. In five tests with soft-tissue samples conducted on the same principle, the percentage recoveries ranged from 92 to 100, and averaged 95.4.

RESULTS OF THE EXPERIMENT

Because of the imposition of the above-described severe experimental conditions, the food consumption of the rats was low and the daily gains in body weight averaged only about 0.9 gm. However, the rats showed no other adverse effects except during the first week of the experiment. At this time

several of the rats, irrespective of the nature of the fluorine supplement received, showed a hematuria lasting 1 or 2 days.

The teeth of the rats were examined once a week during the period of experimental feeding, using a jeweler's lens with a magnification of 4x. Striations of the enamel began to appear during the eighth week of feeding and were visible on the teeth of all rats by the end of the tenth week of feeding. In all probability tooth striations would have appeared earlier if the rats had consumed the experimental diets better. No distinction could be made between the rats receiving CaF_2 and those receiving Na_3AlF_6 with reference to tooth appearance.

In table 1 will be found a summary of the growth data and of the data on fluorine retention. The growth rates of the rats were not affected to an appreciable extent by the nature of the fluorine supplement fed, nor were the appetites of the rat for food, as judged by the numbers of food refusals (not given in the table). The weight of dry, fat-free bone and of dried teeth were also not affected by the difference in fluorine supplement given the paired rats.

The fluorine intake of the rats was equivalent on the average to 13.0 parts per million of solid food consumed, or 0.75 mg. per kilogram of body weight daily. Seventy-seven per cent of the fluorine consumed was contained in the drinking water and 23% in the basal diet.

The concentration of fluorine in the bones averaged 620 parts per million of dry, fat-free substance, and that in the teeth 245 p.p.m. of dry substance. The observed concentration of fluorine in the soft tissues was not significant because of the variable amounts of condensed water in these samples. The weights of the samples may be estimated, however, from the empty weight of carcass, the weight of dry fat-free bones and the dry weight of teeth. From another series of twelve rats, averaging 220 gm. in weight, the total dried, fat-free skeleton averaged 50.6% of the fresh weight. The teeth were assumed to contain 14% of moisture (Matsuda, '27). Using these factors and the average fluorine content

TABLE 1

The results on growth and fluorine retention

Pair number and sex	1 ♀		2 ♀		3 ♀		4 ♂		5 ♂		6 ♀	
Rat number	1	2	3	4	5	6	7	8	9	10	11	12
Fluorine compound fed	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite
Period of feeding, days	97	97	98	98	97	97	97	97	99	99	100	100
Total food consumed, gm.	636	636	768	768	599	599	665	665	502	502	646	646
Total gain in body weight, gm.	61	80	68	72	68	69	110	111	75	80	82	77
Final empty body weight, gm.	134	152	113	119	125	129	162	163	122	127	135	131
Total weight of bones,[1] gm.	7.522	7.367	7.216	7.261	6.554	7.489	7.995	7.435	6.676	6.458	7.930	6.755
Total weight of teeth,[2] gm.	0.399	0.400	0.374	0.382	0.366	0.367	0.356	0.363	0.344	0.354	0.367	0.362
Fluorine in bones, p.p.m.	649	645	634	630	646	590	743	795	573	582	570	700
Fluorine in teeth, p.p.m.	294	261	233	255	250	221	295	255	215	196	188	249
Fluorine ingested in food, mg.	1.909	1.909	2.304	2.304	1.797	1.797	1.997	1.997	1.508	1.508	1.938	1.938
Fluorine ingested in water, mg.	7.153	7.154	5.446	5.447	5.691	5.690	7.877	7.877	4.475	4.475	6.044	6.044
Total fluorine ingested, mg.	9.062	9.063	7.750	7.751	7.488	7.487	9.874	9.874	5.983	5.983	7.982	7.982
Fluorine in bones, mg.	4.882	4.752	4.575	4.574	4.234	4.419	5.940	5.911	3.825	3.759	4.520	4.728
Fluorine in teeth, mg.	0.117	0.104	0.087	0.097	0.092	0.081	0.105	0.092	0.074	0.067	0.069	0.090
Fluorine in soft tissues, mg.	0.127	0.132	0.083	0.071	0.077	0.065	0.136	0.126	(0.081)	(0.475)	0.111	0.098
Total fluorine in carcass, mg.	5.128	4.988	4.745	4.742	4.403	4.565	6.181	6.129	3.980	4.304	4.700	4.916
Estimated fluorine retention,[3] mg.	5.009	4.873	4.673	4.667	4.312	4.469	6.098	6.046	3.905	4.229	4.615	4.830
Per cent retention of fluorine	55.3	53.8	60.3	60.2	57.6	59.7	61.8	61.2	65.3	70.7	57.8	60.5
Per cent of retained fluorine in bones	95.2	95.3	96.4	96.4	96.3	96.8	96.1	96.4	96.1	...	96.2	96.2

TABLE 1—*Continued*

Pair number and sex	7 ♀		8 ♂		9 ♂		10 ♂		11 ♂		12 ♂		Averages	
Rat number	13	14	15	16	17	18	19	20	25	26	27	28	Odd	Even
Fluorine compound fed	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite
Period of feeding, days	103	103	105	105	100	100	100	100	96	96	95	95	99	99
Total food consumed, gm.	756	756	652	652	642	642	684	684	537	537	628	628	643	643
Total gain in body weight, gm.	83	75	79	63	125	120	127	106	112	126	99	95	91	90
Final empty body weight, gm.	158	151	152	138	175	171	173	153	162	176	154	152	147	147
Total weight of bones,[1] gm.	8.703	8.180	8.183	8.746	9.172	7.562	8.570	7.976	8.798	8.511	7.147	8.368	7.872	7.676
Total weight of teeth,[2] gm.	0.370	0.434	0.433	0.392	0.382	0.395	0.382	0.364	0.371	0.352	0.354	0.358	0.375	0.377
Fluorine in bones, p.p.m.	627	630	569	575	566	635	655	683	527	540	613	502	614	626
Fluorine in teeth, p.p.m.	306	238	195	269	276	250	271	248	201	209	278	223	250	240
Fluorine ingested in food, mg.	2.270	2.270	1.958	1.958	1.928	1.928	2.054	2.054	1.611	1.611	1.884	1.884	1.930	1.930
Fluorine ingested in water, mg.	8.142	8.142	6.716	6.717	6.554	6.554	7.174	7.174	5.866	5.866	6.118	6.117	6.438	6.438
Total fluorine ingested, mg.	10.412	10.412	8.674	8.675	8.482	8.482	9.228	9.228	7.477	7.477	8.002	8.001	8.368	8.368
Fluorine in bones, mg.	5.457	5.153	4.654	5.029	5.191	4.802	5.613	5.448	4.637	4.596	4.381	4.201	4.826	4.781
Fluorine in teeth, mg.	0.113	0.103	0.084	0.105	0.105	0.099	0.104	0.090	0.075	0.074	0.098	0.080	0.094	0.090
Fluorine in soft tissues, mg.	0.103	0.110	0.093	0.100	0.159	0.131	0.111	0.080	0.121	0.095	0.100	0.089	0.111	0.100
Total fluorine in carcass, mg.	5.674	5.366	4.831	5.234	5.455	5.032	5.828	5.619	4.833	4.765	4.579	4.370	5.028	5.002
Estimated fluorine retention,[3] mg.	5.554	5.245	4.712	5.114	5.375	4.951	5.754	5.544	4.753	4.685	4.491	4.279	4.938	4.911
Per cent retention of fluorine	53.3	50.4	54.3	59.0	63.4	58.4	62.4	60.1	63.6	62.7	56.1	53.5	59.3	59.2
Per cent of retained fluorine in bones	96.2	96.0	96.3	96.1	95.2	95.4	96.3	97.0	96.0	96.5	95.7	96.1	96.0	96.2

[1] Dry, fat-free weight. [2] Weight after drying at 110°C.

[3] The initial fluorine content of the rats was estimated at 1.6 microgram per gram.

of the soft tissues (0.105 mg.), it may be shown that the concentration of fluorine in the soft tissues averaged 0.71 p.p.m.

The nature of the fluorine supplement had no appreciable effect upon these concentrations of fluorine in the tissues. The averages for the cryolite rats and the calcium fluoride rats were not greatly different and among the twelve pairs of rats there was no consistency in this respect. The pairs divided eight to four in favor either of the cryolite (for bone) or of calcium fluoride (for teeth and soft tissues). In such a situation the application of statistical analysis is unnecessary.

The total quantities of fluorine recovered in the carcasses of the experimental rats were quite similar for the rats on the two fluorine supplements, averaging 5.028 mg. for the rats receiving calcium fluoride and 5.002 mg. for the rats receiving cryolite. The amounts of fluorine retained during the experimental feeding period were estimated by deducting from the amounts of fluorine found, the amounts estimated to have been present in the rats at the start of the feeding period. The six check rats killed at this time contained the following amounts of fluorine in micrograms per gram of weight: 2.4, 1.9, 1.5, 1.5, 1.3 and 1.1, averaging 1.6. The initial content of fluorine was estimated at 1.6 micrograms per gram initial weight.

The quantities of fluorine retained during the experimental feeding period averaged 59.3% of the amount fed for the rats receiving the calcium fluoride supplement, and 59.2% for the rats receiving the cryolite. Among the twelve pairs, four showed the greater percentage retention for the cryolite rats, and with three others the difference was less than 1%. The data, therefore, do not reveal any appreciable difference between the rate of retention of fluorine from calcium fluoride and from cryolite.

Over 96% of the fluorine retained by these rats was deposited in the skeleton, and this quota was remarkably constant among the twenty-four rats, the percentage varying

only from 95.2 to 97.0. The remainder of the recovered fluorine was deposited in about equal amounts in the teeth and in the soft tissues, 1.85 and 2.05%, respectively.

CONCLUSIONS

The fluorine in cryolite is no more toxic to growing rats nor is it retained in the body to a greater extent, than the fluorine in calcium fluoride when both are administered in aqueous solution at the rate of 0.58 mg. per kilogram of body weight daily. The appearance of striations in the incisor teeth is equally rapid with both fluorine compounds.

About 96% of the fluorine retained at an intake equivalent to 13 p.p.m. of food consumed is deposited in the skeleton, and the remaining 4% is about equally divided between teeth and soft tissues.

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A COMPARISON OF THE TOXICITY OF FLUORINE IN THE FORM OF CRYOLITE ADMINISTERED IN WATER AND IN FOOD [1,2]

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In the preceding paper it was shown that the fluorine in cryolite is not appreciably different in toxicity for growing rats from that in calcium fluoride (the form in which fluorine probably occurs in most fluorine-bearing waters insofar as it is present in combination), when both compounds are administered in the drinking water at a low level, 0.58 mg. per kilogram daily. The next step in determining whether the fluorine in cryolite (Na_3AlF_6) in the form of spray residue on fruits and vegetables is any more or less toxic than the fluorine in drinking water, the relation of which to malformation of dental enamel has been quantitatively assessed, is a comparison of the toxicity of the same fluorine compound when administered in water and in food.

Dean, Sebrell, Breaux and Elvove ('34) observed that 500 p.p.m. of NaF in the drinking water exhibited in rats a relatively greater toxicity, as evidenced by growth rate and tooth structure, than the same concentration of fluorine in the diet under their experimental conditions. They state further: "Whether this result is due to differences in the total quantity of sodium fluoride ingested, to more complete absorption,

[1] This experiment was made possible by the donation of funds to the University of Illinois by the Aluminum Company of America.

[2] This investigation was conducted under the supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

to a more rapid rate of absorption or to other factors has not been definitely determined." Marcovitch, Shuey and Stanley ('37) concluded that sodium fluoride affects the teeth of rats at a lower concentration in the drinking water (4 p.p.m.) than in the food (7 p.p.m.), and at a still lower concentration in the drinking water (1 p.p.m.) when the food is cooked to dryness in 3.4 times its weight of such water. In the following year Marcovitch and Stanley ('38) reported that rats receiving 4 p.p.m. of fluorine in the drinking water as sodium fluoride retain nearly twice as much of the element as rats receiving 4 p.p.m. of fluorine as cryolite in the diet, admitting that: "This is due in part to the greater consumption of fluorine in the drinking water, but no doubt solubility is also a factor."

Experiments in which the comparison of the toxicity of fluorine in drinking water and in food, is made with animals receiving unrestricted amounts of both, cannot be expected to answer the question whether the method of administration of fluorine per se is a factor. Nor can the results of such experiments be presumed to possess any wide range of applicability in practical human nutrition, in which water and food are both consumed ad libitum. The ratio of the voluntary consumption of water to that of food is subject to such wide variation on account of differences in the character of the food, the environmental temperature, the activity of the subject and possibly the species of animal as well as other factors, that experimental results pertaining to any particular ratio secured under one set of conditions cannot be applied to other ratios and other conditions.

The experiments to be described below were planned to answer the question whether the method of administration of fluorine per se, in water or in food, modified its toxicity as measured by changes in the dental enamel and by its retention in the body. The source of fluorine in both cases was cryolite.

PLAN OF EXPERIMENT

Twelve pairs of young albino rats ranging in weight from 60 to 90 gm. were chosen from litter mates of the same sex

and approximately the same weight. They were fed according to the paired-feeding technic upon a basal diet, consisting of dried beef powder 25%, dried yeast 8%, sucrose 10%, lard 8%, butterfat 7%, cod liver oil 1%, a calcium-free salt mixture[3] 3%, $CaCO_3$ 1%, and starch 37%. This diet contained 0.88 p.p.m. of fluorine.

The even numbered rats in each pair received this ration with enough cryolite added to bring the fluorine concentration to 10 p.p.m. This required 9.1 mg. of F per kilogram of diet. A solution containing 9.1 mg. of F, as cryolite, per liter was made up to supply the fluorine supplement to the odd numbered rats in each pair. Each odd numbered rat received daily as many cubic centimeters of this solution as it received in grams of the basal diet. Hence, the rats in each pair received the same amount of food daily and the same dosage of fluorine as cryolite, one in solid form mixed into its food and the other in aqueous solution. The cryolite used was a synthetic product that is being marketed as an insecticide. It contained 43.7% of fluorine. As originally obtained, it all passed through a 200 mesh sieve, and during the process of mixing with the starch ingredient of the diet, it was further pulverized in a ball mill for 3 or 4 hours.

In order to obtain the maximum effect of the difference in the method of administration of the fluorine, it seemed essential to separate as far as possible the times of consumption of food and of cryolite solution. Hence, the following schedule was followed. The rats were fed at 4 o'clock in the afternoon and were allowed to eat until 7 o'clock. In order to prevent scattering, each day's portion of food was moistened with about 4 cc. of distilled water, but otherwise the rats received no water until 8 o'clock in the morning. The even-numbered rats received redistilled water ad libitum from 8 A.M. to 4 P.M. The odd-numbered rats received their daily quota of cryolite solution at 8 A.M. and, when this was consumed, as much redistilled water as they cared for until 4 P.M.

[3] Containing no fluorine and, besides the ordinary ingredients, cobalt chloride, zinc chloride and copper sulfate.

Fluorine balance experiments were run on all pairs of rats at least once, and twice for four pairs, in the interval from the tenth to the nineteenth weeks of the experiment. Each collection period lasted 14 days, during which time the rat was confined in a Pyrex crystallizing dish, measuring 190 mm. in diameter and 100 mm. in height. The bottom of the collection dish was covered with a circular filter paper for the absorption of urine, over which was placed a fine metal screen for the collection of the feces. The rat was supported on a metal grid with ½ inch mesh, raised about 2 inches above the bottom of the dish by metal supports. The dish was covered by an inverted cylindrical cage made of hardware cloth and standing about 7.5 inches high. The papers for the absorption of urine were removed every other day, moistened with magnesium acetate solution and dried. The dishes and grids were washed with hot redistilled water several times during each collection period. The washings were made alkaline with calcium hydroxide and evaporated to dryness.

At the termination of the collection period, the treated filter papers were placed within the proper beakers containing the residue from the urine washings, and the papers and residue were ashed at 500°C. in an electric muffle furnace. The feces collections were treated with a solution of magnesium acetate, dried and ashed at the same temperature. Fluorine determinations on feces and urine were made according to the micro-method of Armstrong ('36).

In some of the balance experiments, the urine collections were divided into two periods, from 8 A.M. to 5 P.M. and 5 P.M. to 8 A.M., in order to detect differences in the rate of urinary excretion between the rats receiving their fluorine supplement at 4 P.M. (in the food) and those receiving their supplement at 8 A.M. (in the water).

The rats were killed with ether at the end of 19 or 20 weeks of experimental feeding. The empty weight was determined and the carcasses separated into soft tissues, skeleton and teeth as described in the preceding paper. The fluorine was

determined in each of these samples according to the methods previously given.

EXPERIMENTAL RESULTS

The physical condition of the rats, the rate of growth and the appearance of the teeth were observed throughout the feeding period. The growth of the rats was uniformly slow because of the adverse feeding conditions that were of necessity imposed. The average daily gain in body weight was only 0.83 gm. Food refusals were frequent and in eleven of the twelve pairs were more frequent for the rat receiving its cryolite in the water, indicating a definite impairment of appetite by this method of fluorine administration. In the average, the food refusals were more than twice as frequent for the rats receiving the cryolite solution than for those with cryolite in their diet.

Striations appeared on the lower incisor teeth starting with the seventh week of feeding and by the tenth week all of the rats showed this effect. In four pairs of rats, the rat receiving cryolite in water showed tooth striations first, in four pairs the reverse was true, and in four pairs striations were observed at the same time in both rats. Thus, as far as these weekly examinations of the teeth revealed, there was no difference in the time of appearance of tooth striations between the two methods of fluorine administration.

During the first week or so of the experiment about half of the rats receiving fluorine in their drinking water exhibited hematuria that recurred every few days and then disappeared completely. This phenomenon was observed also in the experiments described in the preceding paper, in all of which fluorine was administered in the water. It evidently represents only a temporary effect upon kidney function, albeit an intense effect while it lasts.

The growth data and the results of the fluorine analyses of the tissues are summarized in table 1. Neither the total gains in body weight made by pair mates, nor the attained body lengths (not summarized in the table), indicate any appre-

TABLE 1

Growth data and fluorine analyses of tissues

PAIR NUMBER AND SEX	1♀		2♀		3♀		4♀		5♀		6♀	
RAT NUMBER	108	109	110	111	112	113	114	115	116	117	118	119
CRYOLITE ADMINISTRATION	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water
Feeding period in days	136	136	136	136	141	141	136	136	136	136	142	142
Total food consumed, gm.	905	905	937	937	971	971	816	816	893	893	816	816
Total gain in body weight, gm.	100	99	97	111	123	126	118	92	102	87	79	89
Final empty body weight, gm.	186	184	176	193	198	199	178	155	191	174	153	165
Total weight of bones,[1] gm.	8.830	10.393	9.692	9.252	8.945	8.955	9.113	8.451	9.867	9.762	9.115	8.479
Total weight of teeth,[2] gm.	0.411	0.437	0.408	0.429	0.440	0.466	0.443	0.403	0.427	0.416	0.401	0.402
Fluorine in bones, p.p.m.	397	464	375	463	392	492	376	498	380	426	368	489
Fuorine in teeth, p.p.m.	208	221	235	247	219	225	190	247	227	260	196	235
Total fluorine ingested, mg.	8.82	8.82	9.26	9.26	9.15	9.15	8.03	8.03	8.72	8.72	7.98	7.98
Fluorine in bones, mg.	3.506	4.823	3.635	4.284	3.506	4.406	3.427	4.209	3.750	4.159	3.355	4.146
Fluorine in teeth, mg.	0.085	0.097	0.096	0.106	0.096	0.105	0.084	0.100	0.097	0.108	0.079	0.094
Fluorine in soft tissues, mg.	0.116	0.160	0.094	0.109	0.120	0.129	0.083	0.089	(0.214)	(0.099)	0.057	0.090
Total fluorine in carcass, mg.	3.707	5.080	3.825	4.499	3.722	4.640	3.594	4.398	4.061	4.364	3.491	4.330
Estimated fluorine retention,[3] mg.	3.569	4.944	3.699	4.368	3.602	4.523	3.498	4.297	3.919	4.225	3.373	4.208
Per cent retention of fluorine	40.5	56.1	39.9	47.2	39.4	49.4	43.6	53.5	44.9	48.5	42.3	52.7
Per cent of retained fluorine in bones	94.6	94.9	95.0	95.2	94.2	95.0	95.4	95.7	92.3	95.3	96.1	95.8

TABLE 1—*Continued*

PAIR NUMBER AND SEX	7♀		8♀		9♀		10♀		11♂		12♂		AVERAGES	
RAT NUMBER	120	121	122	123	124	125	126	127	128	129	130	131		
CRYOLITE ADMINISTRATION	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water
Feeding period in days	142	142	140	140	140	140	135	135	133	133	132	132	137	137
Total food consumed, gm.	853	853	813	813	867	867	900	900	1072	1072	1093	1093	912	912
Total gain in body weight, gm.	81	88	87	89	92	86	100	89	185	181	203	201	114	112
Final empty body weight, gm.	163	168	157	160	154	150	173	160	252	251	279	274	185	186
Total weight of bones,[1] gm.	8.645	9.261	8.752	8.295	10.031	7.721	9.123	8.457	10.541	11.070	12.595	13.165	9.604	9.438
Total weight of teeth,[2] gm.	0.410	0.445	0.437	0.368	0.397	0.397	0.453	0.365	0.431	0.440	0.470	0.418	0.427	0.415
Fluorine in bones, p.p.m.	348	447	347	447	354	472	376	434	336	432	283	365	361	452
Fuorine in teeth, p.p.m.	198	212	179	218	209	212	192	256	227	243	219	251	208	236
Total fluorine ingested, mg.	8.33	8.33	7.92	7.92	8.08	8.08	8.64	8.64	10.10	10.10	10.24	10.24	8.773	8.773
Fluorine in bones, mg.	3.008	4.140	3.037	3.708	3.551	3.644	3.430	3.670	3.542	4.782	3.564	4.805	3.443	4.231
Fluorine in teeth, mg.	0.081	0.094	0.078	0.080	0.083	0.084	0.087	0.093	0.098	0.107	0.103	0.105	0.089	0.098
Fluorine in soft tissues, mg.	0.067	0.082	0.051	0.058	0.068	0.080	0.079	0.083	0.128	0.153	0.153	0.185	0.092	0.107
Total fluorine in carcass, mg.	3.156	4.316	3.186	3.846	3.702	3.808	3.596	3.846	3.768	5.042	3.820	5.095	3.636	4.439
Estimated fluorine retention,[3] mg.	3.025	4.188	3.074	3.732	3.603	3.706	3.479	3.732	3.661	4.930	3.708	4.978	3.517	4.319
Per cent retention of fluorine	36.3	50.3	38.8	47.1	44.6	45.9	40.3	43.2	36.3	48.8	36.2	48.6	40.3	49.3
Per cent of retained fluorine in bones	95.3	95.9	95.3	96.4	95.9	95.7	95.4	95.4	94.0	94.8	93.3	94.3	94.7	95.4

[1] Weight of dry, fat-free bone. [2] Weight of dry teeth.

[3] Total fluorine in carcass minus estimate of initial content of fluorine, taken as 1.6 p.p.m. of initial weight of body.

ciable effect of the method of fluorine administration upon body growth. Also, no consistent differences in the weight of dry, fat-free bone or of the teeth were obtained between pair mates.

However, the fluorine analyses revealed a greater concentration of fluorine in bone and in teeth in all twelve pairs for the rat receiving its cryolite in water. For twelve events, each of which may result with equal probability in either of two ways, a consistent outcome would be obtained by chance on an average of only twice in 4096 trials (from the binomial distribution), or once in 2048 trials. This probability is so small that it may be neglected, and the consistent outcomes observed can reasonably be ascribed to the difference in experimental treatment deliberately imposed, i.e., the method of fluorine administration.

The contents of fluorine in bone and teeth were also consistently greater for the rat in each pair receiving its cryolite via the drinking water, and the same was true with one exception for the fluorine contained in the soft tissues. The one exception occurs in pair no. 5, in which the soft-tissue sample for the rat receiving cryolite in its food exhibited a fluorine content three or four times as large as any other similar sample. It is very probable that this sample was inadvertently contaminated with a small piece of bone, especially since the observed percentage of retained fluorine deposited in the skeleton was much lower for this rat than for any other. It may be concluded, therefore, that equal doses of fluorine cause a greater deposit of fluorine in bone, teeth and soft tissue when administered in the water than when administered in the food.

The estimates of the amounts of fluorine retained by the rats during the experimental feeding period, using the procedure explained in the preceding paper, were less in all pairs for the rat receiving fluorine in its food, the averages being 3.517 and 4.319 mg., amounting to 40.3 and 49.3%, respectively, of the fluorine consumed. Thus, the retention of fluorine was 18.6% less for the rats receiving their fluorine in

the food than for those receiving it in the water. Since the basal diet supplied 9% of the fluorine intake, the observed average difference in fluorine retention must have been produced, outside the limits of random error, by 91% of the fluorine intake of the odd numbered rats. Hence, if these rats had received all of their fluorine in the drinking water, it may be predicted, neglecting random errors, that instead of retaining 0.802 mg. more of fluorine than their pair mates, they would have stored $0.802 \div 0.91 = 0.881$ mg. more, a difference of 20.4, instead of 18.6%. It may be concluded, therefore, that under the conditions of this experiment the administration of fluorine in food, rather than in water, decreases the retention of fluorine, and hence its potential toxicity, by 20.4%.

It is an interesting fact that in nine of the twelve pairs of rats, the rat receiving its fluorine in the water retained a greater percentage of the stored fluorine in its skeleton than its pair mate, the averages being 95.4 and 94.7, respectively. In one pair, the percentages were the same. In making a statistical analysis of this situation, it seemed fair to exclude the results of pair 5 in which a questionably low percentage was obtained, as explained above, for the rat getting its fluorine in the food. For the remaining eleven pairs, the mean difference in the percentage of fluorine stored that was deposited in the bones was 0.418, favoring the rats receiving the cryolite solution, the standard deviation of differences was 0.495, the value of t, 2.656, and the probability, P, that such an average difference was produced by fortuitous factors only, 0.012 (Student, '25). This probability is so small that it may be neglected. We may conclude, therefore, that the greater storage of fluorine in the body associated with its administration in the drinking water occasioned a greater proportional storage in the skeleton.

The results of the fluorine balance trials, summarized in table 2, substantiate the results of the carcass analyses. In thirteen of the sixteen trials, more of the ingested fluorine

TABLE 2

Fluorine balance data. Results expressed on the 14-day basis

PAIR NUMBER AND SEX	1♀		2♀		3♀		4♀		5♀		6♀	
RAT NUMBER	108	109	110	111	112	113	114	115	116	117	118	119
CRYOLITE ADMINISTRATION	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water
Fluorine balance: Wk. 10 and 11												
F ingested, micrograms	915	915	855	855	885	885	820	820				
F in feces, micrograms	228	92	160	95	252	177	109	171				
F in urine, micrograms	270	277	265	270	260	286	244	244				
Per cent ingested F excreted	54	40	50	43	58	52	43	51				
Fluorine balance: Wk. 13 and 14												
F ingested, micrograms									940	940	805	805
F in feces, micrograms									280	158	220	162
F in urine, micrograms									395	488	277	280
Per cent ingested F excreted									72	69	62	55
Fluorine balance: Wk. 18 and 19												
F ingested, micrograms	885	885	980	980	1035	1035	910	910				
F in feces, micrograms	293	224	327	258	388	293	247	184				
F in urine, micrograms												
8 A.M. to 5 P.M.	26	252	148	285	122	280	27	268				
5 P.M. to 8 A.M.	382	95	309	142	352	197	263	110				
Per cent ingested F excreted	79	64	80	70	83	74	59	62				

PAIR NUMBER AND SEX	7♀		8♀		9♀		10♀		11♂		12♂	
RAT NUMBER	120	121	122	123	124	125	126	127	128	129	130	131
CRYOLITE ADMINISTRATION	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water
Fluorine balance: Wk. 13 and 14												
F ingested, micrograms	770	770	835	835								
F in feces, micrograms	222	149	228	146								
F in urine, micrograms	330	330	432	429								
Per cent ingested F excreted	72	62	79	69								
Fluorine balance: Wk. 14 and 15												
F ingested, micrograms					755	755	915	915	1180	1180	1160	1160
F in feces, micrograms					208	129	250	171	356	223	339	209
F in urine, micrograms												
8 A.M. to 5 P.M.					53	152	27	191	99	317	139	317
5 P.M. to 8 A.M.					218	99	211	135	327	165	271	139
Per cent ingested F excreted					63	50	53	54	66	60	65	57

was excreted by the rat receiving its cryolite in the food than by its pair mate receiving its cryolite in the drinking water. On an average, 65% of the ingested fluorine was excreted by the former, and 58% by the latter. It is probably significant that for the first four pairs of rats, upon which two balances were run, a considerably greater percentage of the fluorine intake was excreted for all eight rats during the last 2 weeks of feeding than during the tenth and eleventh weeks. This fact would indicate that the rate of fluorine retention decreased as the feeding period progressed.

Of the fluorine excreted, the greater portion appeared in the urine, but in this regard there was an interesting difference between the two methods of fluorine administration. In fifteen of the sixteen balance trials the percentage of the excreted fluorine appearing in the urine was greater for the rat receiving its cryolite in aqueous solution, the averages being 67 and 57, for administration in water and food, respectively.

The fact that the rats receiving fluorine in the drinking water excreted less of the ingested fluorine than their pair mates, but of what they did excrete more appeared in the urine, suggests that the difference in assimilability of cryolite dissolved in water and that mixed in the solid form (finely pulverized) with food is really a difference in the absorbability from the alimentary canal. An inspection of table 2 will show that the differences in fecal fluorine between paired rats are generally large and, with one exception (pair 4, first balance) favor the rat receiving cryolite in its food. Furthermore, the urinary fluorine excretions on intakes identical for pair mates were very nearly the same, averaging 340 micrograms for the rats receiving fluorine only in the food and 359 micrograms for the rats receiving most of their fluorine in the drinking water. We may reasonably conclude, therefore, that the difference in assimilability of fluorine ingested in water and in food is entirely due to a difference in absorbability from the intestinal tract. However, the poorer ab-

sorbability of the solid cryolite does not necessarily mean that it is only incompletely dissolved by the digestive juices. It may very well be completely dissolved, but its absorption may be hindered by the food residues with which it is mixed.

As was to be expected, the rats receiving their cryolite in the food at 4 P.M. excreted most of their urinary fluorine in the period from 5 P.M. to 8 A.M., while the rats receiving their cryolite in the drinking water at 8 A.M. excreted most of their urinary fluorine in the interval from 8 A.M. to 5 P.M. The percentages for the former rats averaged 80 for the night period, and for the latter 66 for the day period.

DISCUSSION

It is clear from these results that at a low level of fluorine intake equivalent to 10 p.p.m. of food consumed, cryolite administered in the water is retained to a greater extent than cryolite administered in the food and hence is potentially more toxic. In the preceding paper it was shown that no appreciable difference exists between the toxicity or the assimilability of the fluorine in cryolite and CaF_2 administered in the drinking water. On the reasonable assumption that fluorine as it occurs naturally in drinking water is generally present as CaF_2 insofar as it exists in unionized form, it may be concluded that for equal low dosages the fluorine in cryolite consumed with the food, as in fruit spray residues, is less assimilable than the fluorine in drinking water and hence less potentially dangerous by about 20%.

But the consumption of water is several times the consumption of food by weight. Of course the ratio will vary greatly, but a convenient and apparently satisfactory method of arriving at a normal ratio, or a ratio under usual conditions, may be based upon Adolph's ('33) estimate that "roughly a convenient liberal standard of total water intake for any mammal is 1 cc. per Calorie," the latter referring to the energy requirement. This standard agrees well with the estimated normal water intake of an average man weighing 65 kg. of 2400 cc. taken by Richter ('38) from data compiled

by Best and Taylor ('37). It agrees well with Magee's ('37) data from large numbers of institutionalized men and women, the average water intake of whom was about 2600 cc. daily in beverages, fluids and solid food. These figures would approximate the numbers of calories of food energy required by adults. In the case of children, a comparison of the cubic centimeters of water required according to McQuarrie's standards ('33) and the number of food calories required as determined by Maroney and Johnston ('37) approximate each other so closely as to suggest that Adolph's standard of 1 cc. of water per calorie of energy required is of rather general application to all ages.

Taking the fuel value of 1 gm. of dried food in the human diet at 4 calories and the water requirement at 1 cc. per calorie, the normal (or usual) ratio of water consumed to food consumed appears to be about 4 to 1. The proportion of the water intake consumed with the food will, of course, vary greatly. For purposes of calculation we may assume that one-half of the water consumed is taken as a drink and one-half in the food (Newburgh, Johnston and Falcon-Lesses, '29–'30). If twice as much water is drunk as the weight of food eaten, the critical concentration of fluorine in the drinking water defining the upper limit of safety, i.e. 1 p.p.m., would correspond to 2 p.p.m. in the food on an equal dosage basis, or considering also the relative toxicity of equal doses of fluorine in water and in food as determined in this experiment, 1 p.p.m. of fluorine in the water would be the hygienic equivalent of 2.4 p.p.m. in the food.

If all the water in the food were cooking water containing 1 p.p.m. of fluorine, then 1 p.p.m. of fluorine in a domestic water supply would be the hygienic equivalent of 4.8 p.p.m. in the food. Of course this refers to the total intake of solid food, only a small fraction of which would be contained in sprayed fruits and vegetables.

SUMMARY AND CONCLUSIONS

Experiments are reported on twelve pairs of rats designed to measure the relative toxicity of fluorine (as cryolite) when consumed in equal amounts either in food or in water. The feeding experiments were carried out according to the paired-feeding technic, and were supplemented with sixteen fluorine balance trials of 14 days each and analyses of the bones, teeth and soft tissues of all rats at the termination of the feeding period. The fluorine was fed in amounts equivalent to 10 p.p.m. of solid food; 9% of the fluorine intake was contained in the basal diet in unknown forms.

The results of the experiment seem to justify the following conclusions, under the experimental conditions above specified:

1. The method of administration of fluorine at low levels of intake whether in water or in food has no apparent effect on the rate of growth of animals. Administered in the water, however, fluorine definitely depresses the appetite of rats and induces a transient hematuria.

2. No considerable difference in the time of appearance of striations in the lower incisor teeth of rats result from the administration of fluorine in food or in water in low concentration.

3. Doses of fluorine in the food are retained to a less extent in bones, teeth and soft tissues than equal doses of fluorine in the drinking water. Considering the total retention of fluorine in the carcass, this impairment in assimilability amounts to 20%. It seems to be the result entirely of an impairment in absorption from the alimentary tract.

4. Continued ingestion of low concentrations of fluorine in food or in water by growing animals results in a smaller percentage retention in the body.

5. Considering both the difference in the usual consumption of food and of water in practical human nutrition, and the difference in potential toxicity of fluorine in water and in food, a concentration of 1 p.p.m. of fluorine in the drinking water defining the upper limit of safety, is the hygienic equiva-

lent of from 2.4 to 4.8 p.p.m. of fluorine in the total food, depending upon the proportion of the water intake that contains fluorine in the critical concentration.

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AVERAGE VALUES FOR BASAL RESPIRATORY FUNCTIONS IN ADOLESCENTS AND ADULTS [1]

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INTRODUCTION

In preparing a review of the literature concerning physiological changes in adolescence (Shock, '39), the scarcity of adequate data on basal respiratory functions became apparent.[2] Although numerous reports of oxygen consumption have been published, little information on respiratory volume, tidal volume or carbon dioxide and oxygen content of expired air is available either for adolescents or for adults under basal conditions. In such studies the maintenance of basal conditions is important since they can be most easily reproduced and thus allow comparisons between individuals. Repeated respiratory measurements have been made on a limited number of adults (Griffith et al., '29; Hafkesbring and Borgstrom, '27; Hafkesbring and Collett, '24), but such observations do not give us the probable range of values expected in normal subjects. Furthermore, it was found that with the exception of Griffith et al. ('29) in none of these studies were all the respiratory functions measured in the

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[2] Bibliographical references to this literature and to that concerned with basal oxygen consumption have been omitted from this report because of limitation of space.

same individuals. This report is presented to show the range in values of basal respiratory functions in normal adolescents and adults and to indicate changes that take place during adolescence.

METHOD

We have recorded the respiratory rate, the respiratory volume per minute, tidal volume, concentration of oxygen and carbon dioxide in the expired air, oxygen consumption and alveolar carbon dioxide tension in two groups of subjects. The first group consisted of fifty normal boys and fifty normal girls representatively sampled from normal school children, on whom the tests were begun at the ages of 11 or 12 years, and were repeated at 6-month intervals over a period of 5 years.[3] The second group consisted of forty-six normal adult males and forty normal adult females, ranging in age from 27 to 43 years. The average age of the adult males was 27.41 years (with standard deviation of 5.9 years) and of the adult females, 28.75 (S.D. = 5.8). The adults were chosen from staff members and from university students who were presumably healthy, although no systematic medical examination was given. In view of the sedentary activities of these subjects, the average values for metabolic tests may be somewhat lower than for the general population. In the adult group, we used only the tests made in triplicate on 2 successive days, although as many as sixty tests were made on each subject.

Three observations on each subject were carried out on each of 2 successive days as follows: The subject was brought by automobile from his home at 7.30 each morning without breakfast. The laboratory used was on the ground floor of the building so that activity was minimized. While the subject lay on a cot for 20-minute rest period, three determinations of blood pressure and pulse-rate were made by a trained woman assistant. At the end of 20 minutes, a Siebe-Gorman half-mask was tied over the nose and mouth of the subject.

[3] The study of these children is still in progress.

The subject was allowed 10 minutes for respiratory adjustment to the mask and then three 8-minute tests of the basal oxygen consumption were made by the Tissot open-circuit gasometer method,[4] in which the total expired air is collected and the carbon dioxide and oxygen concentration is determined by the Boothby-Sandiford modification of the Haldane technique ('20). All gas volumes were reduced to standard conditions of 0°C. and 760 mm. of mercury. Total respiratory volumes were obtained directly from the gasometer reading, and tidal air volumes were computed by dividing the total respiratory volume in liters per minute by the average respiratory rate per minute. The respiratory rate was counted twice each time for a 1-minute period, during each of the three gas-sampling periods. Samples of expiratory alveolar air were obtained by the Haldane-Priestley technic and were analyzed for carbon dioxide in the Haldane apparatus. Surface areas were derived from the height and weight of the subject by the Du Bois formula ('16).

RESULTS

The average of six determinations of each of the respiratory factors (i.e., three on each of 2 successive days) has been calculated for each subject. These values have been used in compiling frequency distributions for 100 children studied when they were 12, 14 and 16 years old, respectively, and for the ninety-six adults. The mean values with their probable errors are shown in table 1. In this table, the values for total ventilation per minute and for oxygen consumption are divided by the weight or surface area of the subject in order to take into consideration the factor of size of the individual. The reduction in variability which results from this procedure is shown in the values of the coefficient of variations which appears in parentheses in table 1.

Table 2 gives the mean differences between the respiratory functions at the age of 12 and of 14, and the age of 14 and

[4] Each subject was tested on 2 days before the actual experimental series was begun, in order to accustom him to the procedure.

TABLE 1

Mean values for respiratory functions under basal conditions at different age levels

	AGES 11.75–12.24		AGES 13.75–14.24		AGES 15 75–16.24		AGES 18.00–26.99		AGES 27.00–43.00	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Ventilation rate/min.	16.3±0.4 (21.5)	16.1±0.3 (14.5)	17.0±0.2 (14.6)	15.6±0.2 (12.5)	15.6±0.3 (14.9)	15.2±0.3 (13.5)	14.0±0.3 (15.9)	14.7±0.5 (20.8)	13.7±0.3 (14.6)	14.4±0.4 (17.9)
Ventilation volume liters/min.	4.79±0.05 (10.2)	4.54±0.07 (14.4)	5.27±0.07 (13.0)	4.86±0.06 (12.7)	5.13±0.08 (13.3)	4.21±0.08 (13.9)	5.04±0.10 (13.2)	4.45±0.11 (16.2)	5.25±0.09 (11.8)	4.63±0.08 (10.8)
Ventilation volume l./sq.m./min.	3.81±0.05 (11.1)	3.41±0.04 (10.3)	3.55±0.03 (9.5)	3.24±0.04 (12.6)	2.98±0.04 (11.1)	2.67±0.04 (12.7)	2.76±0.05 (13.3)	2.91±0.05 (11.6)	2.93±0.04 (9.2)	2.84±0.06 (14.8)
Tidal volume cc./breath	305±6 (19.4)	289±5 (17.2)	316±6 (18.3)	315±4 (13.2)	344±8 (19.7)	282±6 (16.4)	372±9 (16.2)	319±9 (19.6)	390±7 (13.7)	338±7 (13.4)
Tidal volume cc./sq.m./breath	242±4 (16.2)	216±4 (16.1)	212±3 (13.9)	208±3 (13.2)	200±3 (14.8)	177±4 (16.3)	203±5 (17.8)	202±6 (19.9)	218±4 (12.7)	202±4 (11.7)
Expired air % O_2	16.99±0.03 (1.8)	6.90±0.03 (1.5)	16.84±0.03 (1.9)	16.97±0.03 (1.7)	16.35±0.05 (2.4)	16.59±0.07 (3.1)	16.48±0.05 (2.8)	16.98±0.06 (2.4)	16.90±0.05 (1.9)	17.08±0.05 (2.0)
Expired air % CO_2	3.49±0.03 (7.6)	3.57±0.03 (5.4)	3.58±0.02 (6.7)	3.51±0.03 (7.3)	4.01±0.04 (7.6)	3.81±0.04 (8.9)	3.8±0.05 (9.7)	3.41±0.05 (9.8)	3.53±0.04 (8.9)	3.32±0.04 (8.6)
Alveolar air CO_2 tension	41.0±0.3 (6.7)	40.1±0.2 (5.4)	42.2±0.2 (5.8)	39.4±0.3 (8.3)	42.1±0.4 (7.8)	38.8±0.3 (5.8)	43.0±0.5 (5.1)	41.6±0.6 (8.1)	42.7±0.06 (8.8)	40.0±0.5 (7.8)
Oxygen consumption cc./min.	195±2 (11.7)	189±3 (13.7)	223±3 (13.1)	198±2 (11.6)	244±3 (11.5)	187±3 (10.9)	232±3 (8.4)	187±3 (9.2)	218±3 (9.5)	186±2 (8.9)
Oxygen consumption cc./kg./min.	5.15±0.06 (11.0)	4.46±0.06 (12.1)	4.65±0.05 (10.0)	3.90±0.04 (11.4)	4.13±0.04 (9.2)	3.43±0.04 (9.2)	3.43±0.05 (10.9)	3.38±0.05 (10.5)	3.39±0.05 (9.7)	3.13±0.07 (15.1)
Oxygen consumption cc./sq.m./min.	154.5±1.6 (9.6)	140.8±1.1 (7.5)	149.7±1.3 (8.9)	130.1±1.0 (7.9)	141.8±1.4 (8.6)	117.9±1.2 (7.6)	125.9±1.2 (6.8)	118.3±1.5 (8.2)	122.1±1.0 (5.6)	112.5±1.8 (10.5)
Heat production Cal./sq.m./hr.	45.03±0.46 (9.3)	40.99±0.32 (7.2)	43.46±0.35 (8.5)	37.96±0.29 (8.0)	41.13±0.38 (8.0)	34.29±0.34 (7.7)	36.57±0.38 (7.3)	34.28±0.40 (7.7)	35.44±0.28 (5.6)	32.63±0.50 (10.1)

Figures in parentheses are coefficients of variation.

and 16, in boys and girls. The significance of the age differences has been assessed by computing the ratio between the mean difference in the 100 subjects and its probable error (critical ratio). For these observations, the probable error of the mean difference was calculated from the actual distribution of differences, since each subject was measured at all three ages considered. The significant values in table 2, which indicate residual growth, appear in bold-faced type.

We have found that in boys there is an increase in the absolute minute respiratory volume (which is due to an increase in body size) between the ages of 12 and 14 years. In boys the respiratory rate decreases between the ages of 14 and 16, the tidal volume increases, the concentration of oxygen in the expired air decreases and the expired carbon dioxide increases. In girls, there is an increase in the total respiratory volume between the ages of 12 and 14 years. The changes in the composition of the expired air in girls from 14 to 16 years of age are similar to those in boys, but are not so sharply defined. The average oxygen consumption per minute increases from the age of 12 to 16 years in both sexes. However, when the oxygen consumption is measured as cubic centimeters of oxygen consumed per kilogram per minute or as calories of heat produced per square meter per hour, there is a decrease.

The statistical methods suitable for a series in which repeated observations are made on the same individuals cannot be applied to a series in which the observations are made on different individuals. For this reason, the methods used in comparing the adolescents at the ages of 12, 14 and 18 years could not be employed in comparing the 16-year-old group with the adults. This latter comparison was made by computing the probable error of the difference from the formula.

$$\text{P.E.}_{\text{Mn diff.}} = \sqrt{\text{P.E.}^2_{\text{Mn}_1} + \text{P.E.}^2_{\text{Mn}_2}}$$

The results are shown in table 3. Sixteen-year-old girls are similar to adults with respect to most respiratory functions.

Sixteen-year-old boys differ from adult males in that they breathe more rapidly, have a smaller tidal volume, a higher concentration of oxygen and lower concentration of carbon dioxide in the expired air, and a lower oxygen consumption or heat production.

TABLE 2

Significance of age trend in basal respiratory functions. Re-examination of same 100 children

	CHANGES BETWEEN THE AGES OF 12 AND 14 YEARS 14–12				CHANGES BETWEEN THE AGES OF 14 AND 16 YEARS 16–14			
	Girls		Boys		Girls		Boys	
	Mn diff	C.R.	Mn diff	C.R.	Mn diff	C.R.	Mn diff	C.R.
Ventilation rate/min.	−0.40	1.8	+ 0.56	1.3	−0.53	3.0	−1.64	6.8
Ventilation volume liters/min.	+0.34	6.2	+0.51	7.7	−0.52	7.0	−0.09	1.5
Ventilation volume l./sq.m./min.	−0.15	2.7	−0.29	6.5	−0.50	8.9	−0.57	15.9
Tidal volume cc./breath	+26.0	5.3	+15.0	2.5	−23.7	5.0	+30.1	6.8
Tidal volume cc./sq.m./breath	−9.97	2.8	−29.1	6.5	−24.1	7.0	−12.6	4.7
Expired air % O_2	+0.06	1.3	−0.19	5.2	−0.42	7.1	−0.51	11.2
Expired air % CO_2	−0.04	1.2	+0.12	4.7	+0.35	9.7	+0.43	12.8
Alveolar air CO_2 tension	−0.48	1.2	+1.69	5.2	+0.84	2.7	+0.04	0.1
Oxygen consumption cc./min.	+11.0	5.3	+29.1	12.5	−2.3	1.3	+23.8	8.9
Oxygen consumption cc./kg./min.	−0.57	13.5	−0.54	10.4	−0.41	8.7	−0.54	12.3
Oxygen consumption cc./sq.m./min.	−10.3	8.2	−5.6	3.6	−9.0	7.7	−7.2	5.3
Heat production Cal./sq.m./hr.	−2.88	8.3	−1.70	4.0	−2.73	7.9	−2.24	5.5

C.R. = critical ratio, calculated as $\frac{\text{Mn diff}}{\text{PE}_{\text{Mn diff}}}$

Values greater than 4.0 indicate significant differences.

TABLE 8

Significance of age difference in basal respiratory functions

	16–20				20–35			
	Male		Female		Male		Female	
	Difference	C.R.	Difference	C.R.	Difference	C.R.	Difference	C.R.
Ventilation rate/min.	+1.6 ± 0.42	3.8	+0.5 ± 0.53	0.9	+0.3 ± 0.42	0.7	+0.3 ± 0.60	0.5
Ventilation volume liters/min.	+0.09 ± 0.12	0.8	−0.25 ± 0.13	1.9	−0.21 ± 0.13	1.6	−0.17 ± 0.13	1.3
Ventilation volume l./sq.m./min.	+0.23 ± 0.07	3.3	−0.25 ± 0.07	3.6	−0.16 ± 0.06	2.7	+0.07 ± 0.08	0.9
Tidal volume cc./breath	−28.0 ± 11.70	2.4	−36.8 ± 11.14	3.3	−18.60 ± 11.37	1.6	−19.5 ± 11.61	1.7
Tidal volume cc./sq.m./breath	−2.9 ± 6.23	0.5	−24.8 ± 7.13	3.5	−15.2 ± 6.45	2.4	0 ± 7.03	0
Expired air % O_2	−0.13 ± 0.07	1.9	−0.39 ± 0.09	4.3	+0.41 ± 0.07	5.8	−0.10 ± 0.08	1.3
Expired air % CO_2	+0.21 ± 0.06	3.50	+0.41 ± 0.07	5.9	+0.27 ± 0.07	3.9	+0.09 ± 0.07	1.3
Alveolar air CO_2 tension	−0.85 ± 0.60	1.4	−2.74 ± 0.70	3.9	+0.35 ± 0.73	0.5	+1.61 ± 0.82	2.0
Oxygen consumption cc./min.	+11.8 ± 4.30	2.7	+0.7 ± 3.70	0.2	+13.9 ± 4.01	3.5	+0.50 ± 3.57	0.1
Oxygen consumption cc./kg./min.	+0.70 ± 0.07	10.0	+0.05 ± 0.07	0.7	+0.04 ± 0.07	0.6	+0.25 ± 0.09	2.8
Oxygen consumption cc./sq.m./min.	+15.9 ± 1.9	11.8	−0.4 ± 1.9	0.2	+3.8 ± 1.5	2.5	+5.8 ± 2.3	2.5
Heat production Cal./sq.m./hr.	+4.56 ± 0.54	8.4	+0.01 ± 0.52	0.2	+1.13 ± 0.47	2.4	+1.65 ± 0.64	2.6

C.R. critical ratio, calculated as $\frac{\text{Mn diff}}{\text{PE}_{\text{Mn diff}}}$

DISCUSSION

It is interesting that Griffith et al. ('29), Hafkesbring and Borgstrom ('27), and Hafkesbring and Collett ('24), found respiratory volumes which, if reduced to standard conditions of temperature and barometric pressure, fall within the range of our subjects of the same age and sex. The same groups of authors also reported carbon dioxide and oxygen contents of expired air similar to those in our series. A comparison of the respiratory volumes of our subjects with those of Trumper ('29), however, indicates that the individuals whom he studied were hyperventilating. This difference may be accounted for by the fact that our subjects were trained by repeated testings.

The average values for alveolar CO_2 tension which we have obtained are significantly higher than those reported by Griffith et al. ('29), for subjects of a comparable age. Our values are also higher than those reported by Radsma, Streef and Klerks ('33) in a group of ninety-five adults tested in the tropics. We are not prepared to say whether or not this difference can be attributed to climatic factors. Berconsky and Rossignoli ('32) also obtained slightly lower averages on a group of nineteen adult females. Our values are also slightly higher than those usually quoted in the literature from Fitzgerald and Haldane ('04), although it should be pointed out that basal conditions were not observed in tests made by Fitzgerald and Haldane. The higher values reported here are in closer agreement with the average values for CO_2 tension in arterial blood of 45.9 in fourteen males and 43.2 in seven females found by Shock and Hastings ('34).

Our average values for basal oxygen consumption of boys are, like those reported by Bierring ('31), 7 to 10% lower than the extensive series reported by Boothby, Berkson and Dunn ('36). This discrepancy may be due to the training period used in both our experiments and those of Bierring, although the number of cases in our series exceeds that of any previous studies made at this age level. In the case of girls our values are again 5 to 8% lower than most of those

reported in the literature, with the exception of the results of Benedict and Hendry ('21). However, their experiments were conducted on sleeping girls under experimental conditions that were quite different from the usual clinical procedure. At the higher age levels Stark ('32), Tilt ('30), McKay ('30) and Coons ('31) have reported values comparable to ours, although the number of subjects tested was smaller.

It is apparent from tables 1 and 2 that although total ventilation volume, total oxygen consumption and tidal volume tend to increase with age, when the factor of increase in physical size is removed these variables decrease as the subject grows older. Examination of the coefficients of variation, given in parentheses in table 1, shows also that reducing these measurements by factors involving the size of the body makes the results more homogenous. This decrease in variability as expressed by the coefficients of variation (shown in table 1) is not large in certain of the measurements, but the consistency of the trend is significant. It is evident that calculated surface area is a better measurement of body size than is body weight alone, probably because, in adults especially, body weight is influenced often by increased water and fat without an increase in metabolizing protoplasm.

In measuring the respiratory functions as described above, we have considered that values lying within $\pm$ two times the probable error of the mean ($P.E._{Mn}$) may be regarded as normal, that values lying within ± 3 $P.E._{Mn}$ are high or low normals, and that values ± 4 $P.E._{Mn}$ are definitely abnormal.

In this way it is possible to use these mean values as standards in order to determine whether or not an individual is hyperventilating. Hyperventilation will result in a) increased respiratory volume per minute or per square meter per minute, b) increased tidal volume per breath or per square meter per breath, c) lowered concentration of carbon dioxide and increased concentration of oxygen in the expired air and d) lowered alveolar carbon dioxide tension.

SUMMARY

We have tested a group of fifty male and fifty female adolescents, and a group of forty-six adult males and forty adult females; and have recorded the respiratory rate, the respiratory volume per minute, the tidal volume, the oxygen and carbon dioxide concentrations in the expired air, the oxygen consumption and the alveolar carbon dioxide tension. We have found evidence for growth changes over the adolescent period in certain respiratory functions, such as CO_2 and O_2 content of expired air, alveolar CO_2 tension and tidal volume, which have not been reported before. These measurements may serve as normal figures in the age groups from 10 to 20 years. We have pointed out some of the criteria of hyperventilation.

CONCLUSIONS

1. In boys, the minute respiratory volume increases between the ages of 12 and 14 years, due to an increase in body size.

2. In boys, the respiratory rate decreases between the ages of 14 and 16, the tidal volume increases, the concentration of oxygen in the expired air decreases, and the expired carbon dioxide increases.

3. In girls, the respiratory volume and tidal volume increase between the ages of 12 and 14 years.

4. The composition of the expired air in girls changes between the ages of 14 and 16 as it does in the boys, but the change is not so clearly defined. In boys there is a significant increase in alveolar CO_2 tension between the ages of 12 and 14 years. No significant change in alveolar CO_2 tension was found in girls.

5. The average oxygen consumption per minute increases from the age of 12 to 16 years in both sexes. In respect to body size there is a decrease in oxygen consumption.

6. Most respiratory functions of 16-year-old girls are similar to those of adult females.

7. Sixteen-year-old boys breathe more rapidly, have a smaller tidal volume, higher concentration of oxygen and lower concentration of carbon dioxide in the expired air and a lower total oxygen consumption than adult males.

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DIETARY REQUIREMENTS OF THE GUINEA PIG WITH REFERENCE TO THE NEED FOR A SPECIAL FACTOR[1]

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Study of the nutritional requirements of guinea pigs has been handicapped by the difficulty encountered in inducing these animals to eat synthetic rations. Differences of habit and anatomy make the form and consistency of the diet of far greater importance to guinea pigs than to rats. Goettsch and Pappenheimer ('31), Madsen, McCay and Maynard ('33, '35) and Hogan and Ritchie ('34) have reported experiments with guinea pigs on purified diets. The present communication deals with such experiments and with evidence which, in addition to that recently adduced by Kohler, Elvehjem and Hart ('38) indicates the existence of a distinct dietary factor required by guinea pigs.

[1] Aided by grants from the Board of Research and the College of Agriculture of the University of California, Rockefeller Foundation of New York City, and Merck and Company, Inc. The following materials were generously contributed: thiamin (betaxin) and riboflavin by Winthrop Chemical Co., Inc., vitamin B_1 concentrate by Merck and Co., Inc., wheat germ by General Mills, Inc. and brewers' yeast by The Vitamin Food Co.

EXPERIMENTAL

The diets used were as follows:

	797	800	804
Casein (alcohol extracted)[2]	22	..	..
Casein (commercial)	..	24	24
Sucrose	70	64	50
Lard	..	..	14
Salt mixture (McCollum 185)	4	4	4
Agar	4	4	4
Cod liver oil	..	2	2
Wheat germ oil	..	2	2
Yeast extract[3]	..	≎ 10 parts brewers' yeast	≎ 10 parts brewers' yeast

[2] Acid precipitated casein subjected to two 24-hour extractions with cold 66% (by volume) alcohol, followed by extraction with 95% alcohol to facilitate drying.
[3] Prepared by the method of Richardson and Hogan, '36.

Diet 797 was prepared for feeding by mixing the casein, sucrose and salts and adding the mixture gradually to boiling distilled water (200 cc. water to 1 kg. dry mixture) with constant stirring. When about three-quarters of the mixture was dissolved, one-half the agar was stirred in. After removal from the fire the remaining dry mixture was added and, finally, the remainder of the agar. The rapidly thickening diet was poured out on a slab to cool.

This diet was supplemented with:

a. Three cubic centimeters orange juice, 1.5 gm. brewers' yeast, 4 drops each of cod liver oil, wheat germ oil and ethyl linoleate daily.

b. Five cubic centimeters orange juice, 1.5 cc. yeast extract, 3 gm. brewers' yeast, 4 drops each of cod liver oil and wheat germ oil daily.

Diets 800 and 804 were prepared similarly, but the casein, salts and yeast extract were mixed separately and added to the cooling syrup.

The guinea pigs were kept on shavings. Water was always available. Except when fed by medicine dropper or stomach tube, all supplements were placed in individual feeding compartments.

In a preliminary experiment five guinea pigs, 24 to 30 days old, were placed on diet 797 plus supplements *a* (a diet found to be completely adequate for rats when one-half the quantity of supplements was given). Although the diet was eaten readily at first, the animals failed rapidly. After a month two of the guinea pigs had died and the remaining three were weak and in poor muscle tone. They ate almost none of the basic diet and were losing interest in their supplements. The fur was generally dull and rough, with loss of hair from the upper sides.

On the thirtieth day of the experiment, the surviving animals were given 30 gm. (fresh weight) of the outer leaves of lettuce. (The heavy mid-rib was not weighed nor fed). Within a few hours supplements which were virtually refused the day before were eaten greedily. Consumption of the basic ration increased greatly. Within 3 days the previously listless guinea pigs had energy enough to run about their cages and whistle loudly. They were continued on the experiment for 145 days, at which time they had gained an average of 344 gm.

There seemed two possible explanations of the results: 1) that lettuce contained a hitherto unrecognized factor necessary for guinea pigs but not for rats; or 2) that it increased one of the known factors already present above the minimal level required by this species. The lack of symptoms similar to those arising from deficiency of the known fat soluble vitamins seemed to indicate that the fat-soluble vitamins were not concerned. The animals showed no visible signs of scurvy. Goettsch and Key ('28) and Eddy ('29) have shown that 3 cc. of orange juice daily would supply sufficient vitamin C. Direct evidence that vitamin C was not concerned was afforded when the amount of orange juice was doubled on the twenty-fifth day without alleviating the deficiency. The ineffectiveness of orange juice also shows that vitamin P [4] was

[4] Zilva ('37) was unable to repeat the experiments which Béntsath, Rusznyák and Szent Györgyi ('36) made on guinea pigs and questions the existence of vitamin P in connection with the nutrition of this species.

not the missing factor. There remained only the vitamin B complex to consider. This was supplied in the supplement by 1.5 gm. of brewers' yeast. At the start of the experiment we had intended to feed twice this quantity, but it was only with difficulty that our animals could be induced to eat even as much as 1.5 gm.

Owing to the refusal of the animals to eat larger quantities of yeast and the difficulty of training them to eat from supplement dishes, a yeast extract was substituted for yeast. In this way the supplements could be fed by medicine dropper. Guinea pigs placed on diet 797 failed just as quickly when supplements *b* were supplied as they had with supplements *a*.

In order to make certain that we were dealing with a specific need of guinea pigs whether quantitative or qualitative, not shared by rats, we performed feeding tests with rats. Six female rats placed at weaning on diet 797 plus supplements *b* in one-half the quantity given to guinea pigs, averaged 170 gm. in weight after 8 weeks. The rats were continued on the diets for 6 months and remained in good condition.

The possibility that the quantitative need for vitamin B_1 was not supplied in the yeast or yeast extract presented itself. Hence it was thought advisable to check the question directly. Six litters of guinea pigs 25 days old were segregated into five groups. They were placed on diet 797 plus supplements *b* for 10 days, at the end of which time they had lost an average of 36 gm. One of these groups was then given 30 gm. of the fresh outer leaves of lettuce. A second group received similar portions of lettuce which had been heated on open trays in a steam chamber for 1 hour at 100°C. There were no significant differences between the gains made by the guinea pigs receiving the fresh lettuce and those receiving the heated lettuce.

A third group received an aqueous extract of lettuce (LC2) $\backsimeq$ 25 gm. fresh lettuce. The extract was prepared by extracting 1 kg. freshly ground lettuce with 10 liters of distilled water at room temperature, followed by 12 hours at 2°C. The

filtered extract was concentrated under reduced pressure (temperature 35° to 40°C.) to a thick syrup (volume 20 cc.).

The guinea pigs receiving this extract made gains comparable with those receiving the fresh and the heated lettuce. The fourth group received 800 micrograms of a highly purified B_1 concentrate from rice polishings. This concentrate, when assayed at 400 micrograms with rats depleted of vitamin B_1 on the Sherman-Chase diet, produced a gain of 115 gm. (average of four females) in an 8-weeks' test period. The guinea pigs received the vitamin B_1 rich yeast extract as well.[5] The animals failed to gain.

The fifth group received 3 gm. of defatted wheat germ. The wheat germ used had been extracted with a high grade petroleum ether. Some of this same extracted wheat germ was used for the preparation of the wheat germ autolysate subsequently fed and used in other experiments in this laboratory and was known to be rich in vitamins B_1 and B_6. These guinea pigs failed to gain.

The results of these experiments are summarized in table 1.

As the feeding of considerable quantities of liquid involved much time and labor, the substitution of rations with incorporated vitamin supplements was attempted.

Diets 800 and 804 supplemented with ascorbic acid gave results identical with diet 797 plus supplements *b*. The presence of lard in diet 804 seemed without effect. The guinea pigs responded quickly when given 40 gm. of fresh lettuce daily (table 2).

Although all the evidence indicated that the factor with which we were dealing was water soluble, it seemed advisable to check this point more thoroughly. Grass (timothy lawn trimmings) was chosen as a starting material (a pilot experiment had demonstrated its growth-stimulating activity).

[5] Four rats, after a preliminary 2 weeks' depletion period on the Sherman-Chase diet, gained an average of 138 gm. in the succeeding 8 weeks on the equivalent of 1 gm. brewers' yeast as extract. The average gain with 20 micrograms of thiamin was 135 gm.

An aqueous extract was prepared by extracting 6 kg. of fresh grass with 30 liters of distilled water for 1 hour at 85° to 95°C. The extract was filtered and concentrated to a thick syrup under reduced pressure (temperature 35° to 45°C.). One cubic centimeter of this extract (GC1) was equivalent to 8 gm. of the fresh grass. This extract was fed by stomach tube to guinea pigs previously depleted on diet 800 plus 5 mg.

TABLE 1

Growth responses of guinea pigs held on purified diet 797 to lettuce, vitamin B_1 concentrate and wheat germ

TEST SUPPLEMENT	NUMBER OF GUINEA PIGS	CHANGE IN WEIGHT 10 DAYS BEFORE TEST SUPPLEMENT	CHANGE IN WEIGHT 10 DAYS AFTER TEST SUPPLEMENT
30 gm. fresh lettuce	5	−29	+20
30 gm. heated lettuce	3	−48	+17
Aqueous lettuce extract (LC2) ≎ 25 gm. fresh lettuce	2	−38	+16
800 micrograms vitamin B_1 concentrate	4	−26	−5
3 gm. defatted wheat germ	2	−53	−18

TABLE 2

Growth responses of guinea pigs held on purified diets 800 or 804 to lettuce

DIET	TEST SUPPLEMENT	NUMBER OF GUINEA PIGS	CHANGE IN WEIGHT 10 DAYS BEFORE TEST SUPPLEMENT	CHANGE IN WEIGHT 10 DAYS AFTER TEST SUPPLEMENT
804	40 gm. fresh lettuce	2	−54	+53
800	40 gm. fresh lettuce	3	−33	+39

ascorbic acid. Significant gains were made when it was fed at a level equivalent to 25 gm. of fresh grass.

An ether extract was prepared from 1500 gm. of air dried grass (moisture content 4.5%). The grass was extracted three times with 25 liters of peroxide-free ether. The extract was filtered, concentrated to about 300 cc., washed with water and finally freed of solvent. The yield was 20.3 gm. of a dark green waxy material melting above 100°C. It was diluted with ethyloleate so that 1 cc. ≎ 80 gm. of fresh grass.

This solution (GC2) melted at about 35°C., and was fed warm with a dropper. The guinea pigs failed to respond to this extract.

These results are summarized in table 3.

TABLE 3

Growth responses of guinea pigs held on diet 800 to grass preparations

TEST SUPPLEMENT	NUMBER OF GUINEA PIGS	CHANGE IN WEIGHT 10 DAYS BEFORE TEST SUPPLEMENT	CHANGE IN WEIGHT 10 DAYS AFTER TEST SUPPLEMENT
Air dried grass $\rightleftharpoons$ 30 gm. fresh grass	3	—25	+32
Aqueous grass extract (GC1) $\rightleftharpoons$ 25 gm. fresh grass	3	—11	+27
Ether extract (GC2) of grass $\rightleftharpoons$ 80 gm. grass	2	—29	—22

As the necessary factor was water soluble and relatively heat stable, the possibility existed that some part of the vitamin B_2 complex was not supplied in sufficient quantity in the yeast extract. Riboflavin and rich sources of B_6 and 'filtrate factor(s)' were then tested. Three guinea pigs depleted on diet 800 plus 5 mg. ascorbic acid for 10 days were given 50 micrograms daily of crystalline riboflavin (lactoflavin, Winthrop). Five days later two of the animals had died. Increasing the dose to 100 micrograms failed to benefit the survivor. Vitamin B_6 was supplied as a wheat germ autolysate (Birch and György, '36) and as a crude water extract of liver. Filtrate factor was given as crude liver extract and as the filtrate after the adsorption of this liver extract on fuller's earth. All extracts were administered by stomach tube.

The liver extract was made by placing 5 kg. of fresh, finely ground hog liver in 5 liters of distilled water. The mixture was heated gradually to 60°C., held at this temperature for 15 minutes, then brought to the boiling point and boiled for 5 minutes. After filtration through cheese cloth, the residue was re-extracted in the same manner using 500 cc. of distilled water per kilo of original liver. The combined filtrates

were filtered through Supercel and concentrated under reduced pressure to 1250 cc. A 750 cc. portion was concentrated to 325 cc. and this designated as crude liver extract.

Filtrate factor was prepared as follows: the remaining 500 cc. of the original liver extract was treated with two volumes of alcohol. The filtrate was freed of alcohol and adsorbed six times with 100 gm. portions of fuller's earth, shaking each time for ½ hour. The filtrate was concentrated to 200 cc.

The wheat germ autolysate was fed daily at a level equivalent to 4 gm. of the original wheat germ for 5 days without

TABLE 4

Growth responses of guinea pigs held on diet 800 to components of the vitamin B_2 complex

TEST SUPPLEMENT	NUMBER OF GUINEA PIGS	CHANGE IN WEIGHT 10 DAYS BEFORE TEST SUPPLEMENT	CHANGE IN WEIGHT TEST SUPPLEMENT 10 DAYS AFTER
50 micrograms riboflavin [1]	3	—58	—35 (1 survivor)
Wheat germ autolysate [2] $\approx$ 4.0 gm. wheat germ	3	—32	—7 (1 survivor)
Crude liver extract $\approx$ 40 gm. fresh liver	3	—26	None survived
Liver filtrate $\approx$ 40 gm. fresh liver	3	—23	None survived
'Synthetic' B complex	3	—31	—12 (1 survivor)

[1] Increased to 100 micrograms on fifth day.

[2] Increased to equivalent of 6 gm. on fifth day.

effect. It was then increased to the equivalent of 6 gm. daily but still failed to alleviate the deficiency. This is six to eight times the amount necessary to produce maximal growth in rats on a vitamin B complex-free diet supplemented with thiamin, riboflavin and filtrate factor, and twenty to thirty times the amount found by Birch, György and Harris ('35) to cure dermatitis and produce gains of 40 gm. in 4 weeks.

Crude liver extract and the filtrate prepared from it after the adsorption of B_1, riboflavin and B_6 by fuller's earth were both fed at levels equivalent to 40 gm. of fresh liver daily.

Neither of these preparations showed activity. In regard to B_6, Birch, György and Harris found the equivalent of 5 gm. of fresh liver daily was sufficient to support maximal growth in rats. This laboratory has found that the same liver filtrate fed at levels equivalent to 5 gm. of fresh liver daily was sufficient to support maximal growth in rats on diets lacking in filtrate factor.

Although tests of the individual members of the B complex had shown these factors to be ineffective in promoting growth in deficient guinea pigs even at high levels, the possibility existed that more than one of the factors was needed in large amount. A 'synthetic' vitamin B complex was made up so that each animal received daily 100 micrograms of thiamin (Winthrop betaxin), 200 micrograms of pure riboflavin (Winthrop lactoflavin) and assayed preparations of wheat germ autolysate equivalent to 8 gm. of wheat germ and liver filtrate equivalent to 20 gm. of liver. The feeding simultaneously of high levels of all the B factors definitely established as necessary for rats did not stimulate growth.

These results are summarized in table 4.

DISCUSSION

Where our work has been similar to that of previous investigators, the results are in general accord. Goettsch and Pappenheimer ('31) and Madsen, McCay and Maynard ('33, '35) reported that most of their animals either refused to eat their synthetic diet or died within a 3 months' period. Only two animals are reported by the first mentioned authors to have lived longer than 45 days. Madsen, McCay and Maynard had fair success when they substituted an 'AD concentrate' for cod liver oil in the diet. Eight out of ten animals survived, and 'several doubled in weight' during 70 days. This study was conducted particularly in reference to the development of muscular dystrophy which invariably occurred in guinea pigs reared on the cod liver oil containing diets. Growth on rations containing AD concentrates was superior to that of cod liver oil and dystrophy developed at a later

period. These investigators used cornstarch as the carbohydrate in their diets.

Preliminary experiments not here reported seemed to indicate that the inclusion of dextrinized cornstarch in the diet enabled guinea pigs to maintain themselves, or even gain for a variable period. Hogan and Ritchie ('34) reported that a cornstarch containing diet including 15% yeast gave rather indifferent growth when fed to guinea pigs, but when 2% tikitiki was added excellent growth was observed. In further preliminary experiments carried out in this laboratory, the inclusion of 15% Northwestern yeast led to definite weight gains. These authors attach considerable importance to the quantity of roughage in the diet. Proof that roughage does not play a part in the deficiency we have described is furnished by the fact that the deficiency could be cured by extracts.

The failure of guinea pigs to grow or maintain themselves on a purified diet, shown to be adequate for rats, seems to be due to their need for a distinct dietary factor (or factors) and not to a quantitative difference between their requirements and those of rats for the established vitamins. That fats per se play a part seems to be largely ruled out by the similar behavior of guinea pigs on diet 804 (containing 14% lard) and diet 800 (fat-low). The fat-soluble vitamins seem definitely excluded. They were supplied at high levels and more important, ether extracts of materials potent in curing the deficiency had no effect. Turning to the water soluble vitamins, the factor under consideration is distinct from vitamin C, for this vitamin was supplied in more than adequate quantities in the deficiency producing diets. Information regarding the necessity of vitamin P for guinea pigs has been conflicting, but all agree that no evidence of such a need appears in animals receiving adequate amounts of vitamin C. The fact that the deficiency appeared in animals receiving abundant C in itself shows that the missing factor cannot be vitamin P. Guinea pigs invariably failed on diets in which the vitamin B complex was supplied as yeast or yeast extract in amounts twice those shown to be adequate for rats. The

feeding of the four definitely established B factors individually in amounts greatly in excess of that required to produce maximal growth in rats, failed to save deficient animals. Feeding these same factors simultaneously at high levels was also without effect. It seems extremely unlikely that the amounts of lettuce or grass necessary to cure the deficiency could contain the quantity of B factors supplied by these concentrates. The unimpaired potency of heated lettuce is further evidence against the identity of the factor with B_1. Yeast and liver, potent sources of the entire vitamin B complex, and wheat germ, an excellent source of B_1 and B_6, are relatively low in the factor necessary for guinea pigs, while lettuce and grass, conceded to be poor in the B complex, have this essential factor in high concentration.

Since this work was completed ('37) Kohler, Elvehjem and Hart ('38) have published conclusions substantially the same as ours. Although they approached the problem from a different viewpoint and used a diet of mineralized winter milk entirely unlike the diets described here, our findings are in accord on all points. There would seem every reason to believe that we are both dealing with the same factor.

SUMMARY

1. A highly purified diet which is readily eaten by guinea pigs is described.

2. This diet failed to support guinea pigs, although supplemented with abundant orange juice and sources of the other vitamins in quantities twice those required by rats fed on this diet.

3. If small quantities of lettuce are included in the supplement, guinea pigs will grow normally for considerable periods.

4. The factor present in lettuce is also found in grass.

5. It is water soluble, fairly stable to heat (100°C. for 1 hour) and drying.

6. This factor appears distinct from the known water soluble vitamins for the latter were fed in large amounts without alleviating the deficiency.

7. The factor appears to be identical with that described by Kohler, Elvehjem and Hart.

We wish to thank Prof. Herbert M. Evans for his suggestion of the theme of these experiments and his kind interest.

ADDENDUM

After this paper was sent for publication a communication appeared by Kohler, Randle, Elvehjem and Hart ('39), who found that two unknown factors were necessary for guinea pigs when maintained on purified diets, namely, the grass juice factor and a factor necessary for the maintenance of a normal stomach lining. Grass was found to be high in the former, low in the latter.

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THE MINIMUM VITAMIN A AND CAROTENE REQUIREMENT OF THE RAT [1]

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Previous communications from this laboratory (Guilbert and Hart, '35; Guilbert, Miller and Hughes, '37) presented data on minimum vitamin-A requirements of cattle, sheep, and swine. The minimum was defined as the amount fed daily that just prevented night blindness; and the evidence indicated this amount to be a physiological minimum as regards growth, general well-being, and freedom from detectable clinical symptoms. Little or no storage occurred at these levels.

After these experiments a quantity of the same batch of oil used with cattle, sheep, and swine was saturated with CO_2 and stored at —10°C. for future reference. This cod liver oil and a sample of U.S.P. reference cod liver oil have now been utilized in parallel experiments with rats, thus enabling us to interpret previous results in terms of the reference oil. The requirement of the rat has also been studied by means of a technic as similar as possible to that used for larger animals; and the relation of the vitamin A and carotene requirements to body weight has been further tested with this species.

METHODS OF PROCEDURE

Criteria used to determine minimum requirements. Preliminary investigations showed that procedures involving testing of vision are impracticable in the rat. Differences in sensitivity to light as indicated by contraction of the pupil,

[1] This report is part of an investigation on the relation of nutrition to reproduction which became cooperative with the United States Department of Agriculture, Bureau of Animal Industry on July 1, 1929.

could not be detected in deficient animals. Wagman and Gullberg ('38) found, furthermore, by means of an infra-red photographic technic that no significant difference in rate of pupil dilation depending on vitamin-A intake could be demonstrated. We, therefore, adopted the vaginal-smear method, since aberration from normal cyclic changes is the first detectable symptom of vitamin-A deficiency thus far reported for the rat. Changes in the vaginal epithelium of the rat after vitamin-A deficiency, first observed by Evans and Bishop ('22), have served many workers as a basis for determining vitamin A. These changes are described in detail by Mason and Ellison ('35). Although most workers agree that the continued presence of cornified cells in the vaginal smear is a good index of vitamin-A deficiency, some investigators (Coward, '38) find difficulty in estimating slight responses to small doses. In the work presented here we have endeavored to eliminate this difficulty by using groups of rats which had been depleted of vitamin-A stores and which were then treated with daily doses of vitamin A, constant in respect to body weight. The test period was 60 days or longer.

Different subminimal levels produced characteristic variations from the normal vaginal-smear picture during the estrous cycle. These variations from normal were remarkably constant over long periods when the daily dose remained constant with respect to individual body weight. The rhythmic changes of the normal vaginal smear as described by Long and Evans ('22) are designated in this paper by the following abbreviations:

'O,' principally round, nucleated epithelial cells;

'corn,' cornified cells;

'leac,' cornified cells disappearing, leucocytes and epithelial cells with few if any cornified cells;

'le,' scant, 'dry' smear, consisting of leucocytes and epithelial cells with few if any cornified cells.

For convenience in reporting the data we have arbitrarily classified the daily vaginal smear records as follows: Class 1 (normal), o, corn, leac, le, o, corn, leac, le, etc.; class 2, o, corn, corn, leac, o, corn, corn, leac; class 3, corn, corn, leac,

leac, corn, corn, leac, leac; class 4, corn, corn, corn, leac, corn, corn, corn, leac; class 5, continuously cornified cell smears. As the level of vitamin-A intake was lowered, the amount and persistence of cornified cells progressively increased until they appeared continuously and masked the rhythmic changes that may have continued in the ovary. At low levels of intake, regular cycles could be discerned by less copious smears and by the appearance of leucocytes and epithelial cells at (apparently) the diestrous interval even though cornified cells did not entirely disappear. The lowest level of vitamin-A intake that maintained a normal vaginal-smear picture with a scant diestrous smear containing practically no cornified cells is considered the minimum in this investigation.

Though the classification just given is arbitrary and though only 4-day cycles are represented, there was no difficulty in classifying animals with 5- and 6-day cycles. Individual vaginal-smear records were scored according to this classification, and these were averaged to obtain a numerical expression for the vaginal-smear record for each group.

Analysis of cod liver oils. Unsaponifiable fractions of both the U.S.P. reference oil and no. 760, the oil used in the previous experiments, were analyzed at the beginning and the end of the experiments with a Hilger Vitameter A. Holmes and Corbet ('37) reported the isolation of crystalline vitamin A having a value for $E^{1\%}_{1\,cm.} = 2100$. The following tabulation gives the vitameter determinations on both oils, using first $E^{1\%}_{1\,cm.} = 1600$, the value assigned by Carr and Jewell ('33), and then $E^{1\%}_{1\,cm.} = 2100$, the value reported for crystalline vitamin A. The figures (1) and (2) following the designation of the oil represent values obtained during the earlier and the present experiments respectively.

COD LIVER OIL	$E^{1\%}_{1\,cm.}$	PER CENT VITAMIN A $E^{1\%}_{1\,cm.} = 1600$	PER CENT VITAMIN A $E^{1\%}_{1\,cm.} = 2100$
U.S.P. Ref. (1)	1.31	0.082	0.062
U.S.P. Ref. (2)	1.32	0.083	0.063
No. 760 (1)	1.32	0.083	0.063
No. 760 (2)	1.35	0.084	0.064

Judging from the results, the potency of oil no. 760 did not change during the various experiments, and the extinction coefficients of this and the U.S.P. reference oil were the same. Our value for the reference oil, $E^{1\%}_{1\ \mathrm{cm.}} = 1.32$, is exactly that found by McFarlan, Reddie and Merrill ('37). Considering the reference oil to contain 3000 I.U. per gram, the conversion factor would then be 2270. This E value is also within the range obtained by various investigators and reported by Irish ('36), Wilkie ('38) and Hume and Chick ('35). Since the physical constant of Holmes and Corbet ('37) is based upon a more highly purified form of vitamin A, we have used the value 0.063% vitamin A for both oils. Dosage is calculated and former results are revaluated on this basis. The cod liver oils were diluted with cottonseed oil for feeding.

Carotene. The crystalline carotene was obtained from the S.M.A. Corporation and was from the stock used in the previous experiments with sheep and swine. It contained about 88% beta and 12% alpha carotene. A stock solution was made containing 0.31% [2] carotene in cottonseed oil. From this stock for daily use, was prepared a more dilute solution containing 0.05 microgram of carotene per milligram of oil. Only enough dilute solution to last about 1 week was made at one time. Colorimetric analysis was made at frequent intervals throughout the experiments, both on the stock solution and on the solution used for feeding. The stock solution was kept in the dark at 15°C.

Dosing of rats. The oil solutions containing vitamin A or carotene were administered daily per os from a 2 cc. hypodermic syringe fitted with a no. 26 needle. The tip of the needle was ground off squarely, and each tip calibrated for delivery of one drop of oil. Repeated weighings of drops of oil showed that the variation in the amount delivered did not exceed ±5%. All levels of dosage were maintained constant with respect to body weight. The rats were weighed every 5 days, and the dosage calculated for the next 5 days for each weight.

[2] Colorimetric determination using dye standard matched against pure beta carotene M.P. 184°C.

Animals used and basal diet employed. During the last 10 days of lactation the stock colony diet of the mother rats was replaced by the vitamin A-free basal diet. The young female rats used in the experiments were placed on the basal diet when they were weaned at 21 days of age. The composition of this diet was as follows:

	%
Cornstarch	67
Casein, heat treated, 7 days at 100°C.	20
Brewer's yeast, irradiated	5
Cottonseed oil	5
Salt mixture no. 351 (Hubbell et al., '37, p. 276)	3
	100

RESULTS

Depletion time. Vitamin-A therapy was instituted when individuals had shown continuous cornification of the vaginal smear for 5 successive days. Control rats receiving no supplement at this time were never observed to return to a normal smear. We refer to the beginning of this period of continuous cornified smear as the time of depletion. Table 1 summarizes the results with 200 rats up to the time of depletion as defined above.

TABLE 1

Summary of 200 rats on basal diet from weaning to time of vitamin-A depletion

	WEIGHT AT 21 DAYS	VAGINAL SMEAR BECAME CONTINUOUSLY CORNIFIED	
		Age	Weight
	gm.	*days*	*gm.*
Mean	46.0	53.1	165
Standard error	±0.32	±0.32	±0.45
Coefficient of variation	9.8%	8.7%	3.9%

Fifteen untreated controls continued to gain weight for an average period of 27 days after the appearance of continuous cornified smears and reached an average weight of 200 gm. Subsequently they developed other typical symptoms of vitamin-A deficiency, declined in weight and died.

Minimum vitamin A and carotene requirement of the rat. Table 2 presents the data on all rats used in these experiments. The minimum level to maintain normal vaginal smears in all rats in a group was, for oil no. 760, between 4.6 and 5.3 µg. per kilogram body weight. Since five out of six rats

TABLE 2

Effect of varying levels of vitamin A and of carotene on vaginal smears of rats

SOURCE OF VITAMIN A	NUMBER OF RATS IN GROUP	NUMBER OF DAYS ON TEST	DAILY INTAKE, VITAMIN A OR CAROTENE, µG. PER KILOGRAM BODY WEIGHT	INDIVIDUAL VAGINAL SMEAR SCORES	AVERAGE VAGINAL SMEAR SCORE
C.L.O. no. 760	6	65	3.0	444333	3.5
	6	73	3.8	322211	1.8
	6	62	4.6	211111	1.2
	6	60	5.3	All normal	1.0
	6	60	6.1	All normal	1.0
U.S.P. ref. oil	6	65	3.0	443333	3.3
	6	73	3.8	221111	1.3
	7	67	4.6	All normal	1.0
	6	60	5.3	All normal	1.0
	6	60	6.1	All normal	1.0
Carotene	14	70	6	44444444333322	3.4
	15	70	8	443333222222222	2.5
	12	104	10	333322222211	2.2
Carotene + bile salts	9	104	10	332221111	1.8
Carotene	15	70	11	322222222221111	1.8
	12	104	13	322222111111	1.6
	15	70	14	222222211111111	1.5
	12	104	15	222111111111	1.2
	6	53	20	All normal	1.0
	6	53	40	All normal	1.0
	6	53	80	All normal	1.0
	5	53	200	All normal	1.0
	6	53	400	All normal	1.0
	6	53	600	All normal	1.0

on the lower level were normal, the minimum level is probably nearer 4.6 than 5.3. In terms of the U.S.P. reference oil the minimum level was between 3.8 and 4.6 µg. per kilogram body weight. Thus the reference oil showed slightly greater potency, although the extinction coefficients were practically

equal. Based on the average group scores, 4.7 and 4.1 μg. per kilogram body weight may be taken as the minimum levels for oil no. 760 and the reference oil respectively. Since the dilutions and amounts of oil fed were the same, the value of oil no. 760 may be calculated as 87% that of the reference oil or 2600 U.S.P. units per gram. At each level of dosing, the average vaginal smear score was slightly lower for the U.S.P. reference oil than for oil no. 760, a fact which probably means that there was more irrelevant absorption at the vitamin-A wave length in the latter oil. On the basis of the reference cod liver oil, the minimum level is 18 to 22 I.U. per kilogram body weight per day.

Table 2 shows that the minimum level in terms of carotene lay between 15 and 20 μg. per kilogram body weight. As Greaves and Schmidt ('35) have demonstrated, desoxycholic acid plays an essential role in the absorption of carotene in the rat. To test the effect of additional bile salt in normal animals, nine rats were given 1% desoxycholic acid in their basal ration and were held during the test period of 104 days on a 10 μg. per kilogram carotene level. Table 2 shows a slightly improved utilization of carotene in these rats.

At autopsy, about 45 days after depletion, no storage was found in the livers of rats receiving the higher levels of vitamin A. On the other hand, some storage was found in rats on levels of 4 to 6 μg. per kilogram that were autopsied after 90 days or more of vitamin-A dosage. Baumann et al. ('34) found no storage after 4 weeks' dosage at approximately twice our minimum level in rats that had been depleted to the stage of incipient xerophthalmia. Approximately four times our minimum level resulted in storage under like conditions. This apparent difference in minimum level to produce storage probably resulted from differences in the degree of vitamin-A depletion of the rats before dosage was commenced and in the length of period of dosage before autopsy was made. We could not demonstrate the storage of carotene in the livers of rats treated with carotene until a daily dose of

80 µg./kg. was reached. This represents four to five times the minimum level to produce a normal vaginal smear.

There was no significant difference in weight gains in any of the various levels of vitamin A or carotene dosage. Control rats, however, before showing weight losses, attained the weight at which the growth curve normally flattens out. The conditions of these experiments are therefore not critical as regards minimum levels to support normal gains. The data do show, however, normal gains at the minimum level that prevented any abnormal degree of vaginal cornification.

Chemical analyses were made upon the carcasses of a number of rats from each group at the end of the test period on various levels of vitamin A. These were compared with analyses of controls taken at the beginning of the test period when vitamin A was depleted. The results revealed no significant differences in the percentage composition of the tissues.

Despite some variation in the character of vaginal smears between animals on the same level of vitamin-A intake, the vaginal-smear picture of individual rats remained remarkably constant after a period of 10 to 20 days following the institution of vitamin-A therapy.

Effect of large single doses of vitamin A. Rats depleted to such a degree that they exhibited 5 days' continuous cornified smears were given single doses at the rate of 150 and 300 µg. per kilogram body weight respectively. The time elapsing before vaginal cornification reappeared was recorded. Several such tests were made on each individual. The results were as follows:

Number of trials	*Rate of dosage µg. per kg.*	*Number of days from dosing to reappearance of vaginal cornification*
15	150	21.6±0.8
10	300	27.6±1.7

As compared with daily dosing, the smaller of the single doses was about 70% efficient, and the larger about 45%. In a series of experiments by Greaves and Schmidt ('36) female rats which had been depleted of vitamin A as judged from the

vaginal-smear picture were given a large single dose of vitamin A (375 units). Their rats varied from 117 to 181 gm. when the dose was given and received protection for 23 to 24 days. This dose is two to four times as great as in our experiments yet the period of protection was about the same. We can offer no explanation for this discrepancy except that the conditions of our experiments were probably not the same as theirs. It seems evident again from these experiments that the amount of vitamin A which can be absorbed and stored from one single dose, is limited. These results, which again confirm the findings of Baumann et al., emphasize the importance of daily feeding of small doses in determining the minimum level of vitamin A and carotene.

DISCUSSION

The minimum vitamin-A intake required to prevent vaginal cornification in the rat was found to be about 4.1 µg. per kilogram for U.S.P. reference cod liver oil and about 4.7 µg. per kilogram for oil no. 760 on the basis of vitameter analyses. These results, converted to biological units on the basis of the reference oil, show the minimum requirement to be 18 to 22 I.U. per kilogram body weight. This coincides with the data presented by Guilbert and Hart ('35) and by Guilbert, Miller and Hughes ('37) when similarly converted. The minimal carotene requirement for the rat was between 15 and 20 µg. per kilogram, an amount slightly lower than that found for domestic animals.

The uniformity of response to definite levels of intake over periods during which body weight and stage of maturity changed significantly, together with the close agreement with previous data, confirms the relation of requirement to body weight.

Recognition and application of this fundamental principle to biological test procedures should still further reduce variability of results. Adaptation of the method of procedure employed in these investigations has promising possibilities for routine biological testing. With a standardized procedure

the test period could be reduced to 4 weeks, and the same animals could be used repeatedly. The method has an advantage over the vaginal smear methods previously used in that responses to small doses can be measured. The advantage over the growth procedure apparently rests on the fact that only earliest manifestations of deficiency are involved, in contrast to more drastic pathological changes that may be present when growth ceases. These conditions undoubtedly contribute to variation in growth response. The disadvantage is that the method is somewhat laborious.

SUMMARY

By means of a vaginal-smear technic and a daily dosing of rats at definite levels with respect to body weight, the minimum level to prevent vaginal cornification was found to be 3.8 to 4.6 μg. or 18 to 22 I.U. per day of vitamin A per kilogram body weight.

Similarly, the minimal carotene level was between 15 and 20 μg. per kilogram body weight. No detectable storage occurred until a level of 80 μg. per kilogram body weight per day was given. Large single doses were less efficiently utilized than daily doses, the efficiency decreasing with increasing size of dose.

Simultaneous administration of bile salts with carotene slightly increased its efficiency.

The results confirmed previous data on the relation of vitamin-A requirement to body weight.

The application of this principle to biological testing in general is discussed, together with the adaptability of the vaginal-smear technic used in these experiments for routine biological testing.

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CONDITIONS AFFECTING THE CONTENT OF CHICK ANTIDERMATITIS VITAMIN IN YEAST [1]

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Yeast is an important source of the B vitamins. It is now recognized as a material rich in thiamine, riboflavin, nicotinic acid and for years has been included in many experimental diets as a source of one or more of these essentials. While yeast may be a good source of the B vitamins, it is apparent that different yeasts vary greatly in their vitamin content. The well-known difference between brewers yeast and bakers yeast is an example in point. The former is usually five times as rich as the latter in vitamin B_1. Likewise some commercial bakers yeasts contain twice as much B_1 as other samples.

The most important factor affecting the B_1 content seems to be the kind of medium in which the yeast is propagated. In a previous publication (Pavcek, Peterson and Elvehjem, '37) it was shown that bakers yeast contained about 10 international units per gram of dry matter when grown in a grain wort medium, but only 5 international units when grown in a molasses-salts medium. The figure fell to about 3 international units when the yeast was produced in a synthetic medium, glucose-salts. These same batches of yeast have now been assayed for their content of the chick anti-dermatitis factor and the results are given herein. Very few assays of yeast have been made with respect to this factor and none in

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

relation to the medium upon which the yeast was grown. The recent demonstration of the relation of pantothenic acid to the chick antidermatitis factor increases the significance of these results.

EXPERIMENTAL

Production of yeasts. The conditions under which the yeasts were grown, such as composition of medium, methods of aeration, period of fermentation, etc., have been described in detail (Pavcek, Peterson and Elvehjem, '37), and hence will not be repeated here. The procedures generally used in commercial practice were followed, and the yields were approximately those obtained in the industry. Likewise the vitamin B_1 content of the experimental yeasts were essentially the same as those of commercial yeasts when similar methods of propagation were employed. We feel, therefore, that the yeasts represent what may be expected when well-recognized methods of production are followed.

Chick assay. Day-old white Leghorn chicks were fed the basal ration, 241H (Mickelsen, Waisman and Elvehjem, '38) which contains all the vitamins known to be required by this species, except the antidermatitis factor. Four chicks were used in a group for each assay. The dried yeasts were added at various levels to the basal ration. If necessary, the assays were repeated until a level was found at which protection was obtained and another at which symptoms of dermatitis appeared in one or more members of the group. The minimum protective level, therefore, lies between these two figures. As a check on the procedure both positive and negative controls were run. The negative controls usually developed severe dermatitis and most of them died in from 3 to 4 weeks. When an occasional bird survived longer than this, the gain in weight was never over 50 gm. in 5 weeks. The feeding period extended over 6 weeks, but the gain in weight made in 5 weeks was taken as most suitable for record. Because of possible variations both in yeasts and in chicks, a considerable number of duplicate batches of yeast were grown and assays repeated.

Duplicate assays on the same batch of yeast agreed very well and chick assays of different batches grown on the same medium showed less variation than might be expected.

Data. Assays of twenty-three experimental batches of yeasts are given in table 1. Those grown in grain-wort were richest in anti-dermatitis factor, the molasses yeasts were next in order and the glucose-salts batches contained the least

TABLE 1

Assay of laboratory-grown yeasts

NUMBER	YEAST	LEVEL FED	AVERAGE GAIN IN 5 WEEKS	NUMBER OF CHICKS SHOWING SYMPTOMS
		%	*gm.*	
Control, 3 lots		0	48[1]	12
Series I. Yeasts grown on glucose-salts medium				
131	S. cerevisiae	4	71	3
131	S. cerevisiae	6	94	0
405	S. cerevisiae	4	65	3
405	S. cerevisiae	6	94	0
212	S. logos	2	88	3
212	S. logos	4	83	1
305	W. anomala	4	54	2
312	E. vernalis	6	122	0
Series II. Yeasts grown on molasses-salts medium				
101	S. cerevisiae	2	125	1
101	S. cerevisiae	4	165	0
406	S. cerevisiae	2	62	3
406	S. cerevisiae	4	112	0
304	W. anomala	4	52	3
313	E. vernalis	4	82	4
Series III. Yeasts grown on grain wort medium				
208B	S. cerevisiae	2	108	2
208B	S. cerevisiae	4	122	0
400	S. cerevisiae	1	49	4
400	S. cerevisiae	2	123	1
400	S. cerevisiae	4	172	0
210B	S. logos	2	117	0
210B	S. logos	4	141	0
302	W. anomala	2	91	0
302	W. anomala	4	135	0
311	E. vernalis	2	64	1

[1] Average for two chicks. Other ten died before the end of 5 weeks.

amount of the factor. The protective levels for the three groups were approximately 2, 4 and 6%. Some variations from these figures were obtained, but these were not greater than would be expected from different batches of yeast and from different lots of chicks. For example: S. logos (no. 212) in the glucose-salts series seemed somewhat better than the other yeasts in this series and gave almost adequate protection at a 4% level. In the molasses group, W. anomala (no. 304) and E. vernalis (no. 313) proved inadequate at 4%.

TABLE 2

Assay of commercial yeasts

NUMBER	YEAST	LEVEL FED	AVERAGE GAIN IN 5 WEEKS	NUMBER OF CHICKS SHOWING SYMPTOMS
		%	*gm.*	
401	Bakers R	2	143	0
401	Bakers R	4	135	0
402	Bakers A	1	115	3
402	Bakers A	2	172	0
402	Bakers A	4	216	0
407	Bakers F	2	112	1
407	Bakers F	4	126	0
316	Brewers P	2	115	2
316	Brewers P	4	150	0
403	Brewers L	2	112	0
403	Brewers L	4	185	0
404	Brewers C	1	52	3
404	Brewers C	2	127	0
404	Brewers C	4	187	0

However, one batch of S. cerevisiae (no. 101) gave very good growth at 2% and only one of the four chicks in the group showed symptoms of dermatitis. The figure, 2%, is perhaps somewhat low for grain-wort yeasts. Slight symptoms of dermatitis appeared in three cases at this level. At 4% there was no evidence of dermatitis, and growth was exceptionally good.

Assays of commercial yeasts are given in table 2. Most, if not all, were grown on a wort medium and the protective level was 2%. This is the same figure as was found for the labora-

tory yeasts, grown on a wort medium. No difference is manifest between bakers and brewers yeasts in respect to the chick factor. This result is in marked contrast to the ratio between the two with respect to vitamin B_1. Brewers yeast usually contains several times as much vitamin B_1 as bakers yeast.

The figure for grain-wort yeasts and commercial yeasts is about the same as that for animal tissues, richest in this vitamin. Liver and kidney protect at a 2% level but some tissues e.g., lean beef, must be fed at a 30% level (Waisman, Michelsen and Elvehjem, '39). .

DISCUSSION

From the recent work of Woolley, Waisman and Elvehjem ('39) it appears that the chick anti-dermatitis factor is pantothenic acid. This compound has been extensively investigated by Williams and associates ('33, '34, '39) who found that yeast growth is greatly stimulated by additions of pantothenic acid to the medium. Although yeast growth is stimulated by pantothenic acid, the data reported here show that pantothenic acid is also a product of yeast growth. The yeast crop on the glucose-salts medium ranged from 353 gm. for batch 305 to 1037 for batch 131. The inoculum, a wort-grown yeast, was equivalent to 50 gm. of dry yeast. Hence the multiplication of cells was from seven- to twentyfold, while the potency decreased only to one-third of the original value. The innoculum, therefore, contained only from 15 to 40% as much pantothenic acid as the yeast crop. A similar situation exists with respect to vitamin B_1, which may be both a stimulant and a product of yeast growth.

SUMMARY

The amount of chick anti-dermatitis vitamin (pantothenic acid) in yeast varies with the medium in which the yeast is grown. Yeast grown in grain wort gave protection when fed at a 2% level, molasses yeast at 4% and glucose-salts yeast at 6%. Commercial bakers and brewers yeasts also protected at

the 2% level, which is about the potency of animal tissues richest in this vitamin.

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INFLUENCE OF MASSIVE DOSES OF VITAMIN B_1 ON FERTILITY AND LACTATION[1]

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Following the numerous reports on the toxic effects of massive doses of vitamin D, it was logical for nutritional investigators to be interested in the accumulation of evidence on the possible existence of other hypervitaminoses. For some years it was planned to study vitamin B_1 from this standpoint but our studies on the biochemistry and pathology of deficiency diseases did not permit following this line of research. The report, however, of Perla ('37) that vitamin B_1 in amounts equivalent to forty times the maintenance requrement produced toxic effects as evidenced by inability of rats to rear their young successfully, stimulated this investigation, which was begun in January of 1938.

Perla used a stock diet consisting of 15 gm. per day of a basic mixture of hominy 100 parts, rolled oats 15 parts, fine meat and bone 25 parts, salts 1½ parts, and dried milk 16 parts, to which was added a few drops of cod liver oil, 0.3 gm. of wheat germ and 0.3 gm. of crude Fleischman's brewer's yeast per rat. He found that supplementing his stock diet with Mead Johnson's brewer's yeast equivalent to 50 I.U. of vitamin B_1 per animal per day resulted in a disturbance in lactation in the first generation which was accentuated in the second generation. The effect was more pronounced in

[1] Research paper no. 620, journal series, University of Arkansas. This is paper XXV in the series Dietary Requirements for Fertility and Lactation.

animals fed a vitamin B_1 concentrate (adsorbate) among which still-births were also common. Synthetic vitamin B_1 was somewhat less toxic than the vitamin B_1 adsorbate but similar interference with lactation occurred in the second generation.

While the work initiated in our laboratory was in progress the monograph on vitamin B_1 (thiamin)[2] by Williams and Spies ('38) appeared in which they criticized the findings of Perla. Since Perla injected the synthetic vitamin B_1 subcutaneously, they state: "The disturbances of very young litters for purposes of daily injections seems a questionable practice which might well lead to the abandonment of the young by the mothers." Furthermore, they refer to experiments of Ammerman and Waterman who administered 80 to 1000 µg. of thiamin daily to each rat as a supplement to a stock diet, which was continued for three generations, and reproduction was perfectly normal. Since no reference is given to this work in the bibliography, the work quoted probably represents unpublished data. Without knowing the composition of the stock diet and in the absence of experimental data, it is impossible to evaluate the investigations of Ammerman and Waterman. But at least it appears that we have conflicting results on the influence of massive doses of vitamin B_1 on lactation efficiency when carried on for several generations. It is therefore, all the more pertinent that the results of this laboratory be reported at this time.

Since 1920 we have employed the Wistar strain of albino rat and have successfully reared our animals on the following stock diet: whole wheat, 27; rolled oats, 26; yellow corn, 25; linseed meal, 15; commercial casein, 5; cod liver oil, 1; NaCl, 0.5; and $CaCO_3$, 0.5. This diet is supplemented 6 days a week with 5 cc. of fresh cow's milk per animal until breeding when it is increased to 10 cc. and during lactation, when the young begin eating, to 15 cc. daily. Each animal also receives 15 gm. fresh lettuce once weekly. On such a ration we raise 95 to 98% of our young, the mortality being less

[2] Vitamin B_1 and thiamin are used interchangeably in this paper.

than 5%, and very seldom do we encounter a case of sterility. When we attempted to remove either the supplementary lettuce or milk from this stock diet, the infant mortality increased to over 50%. Removing both the milk and lettuce increased the infant mortality to 60% accompanied by sterility. Therefore, as a starting point in this research, our stock diet supplemented with milk and lettuce was employed.

EXPERIMENTAL

The rats in this study were divided into four groups: A) each animal received 10 μg. thiamin daily; B) each animal received 100 μg. thiamin daily; C) each animal received 200 μg. thiamin daily, and D) each animal received 400 μg. thiamin daily. The thiamin used was the pure crystalline product of Merck.[3] The results are summarized in table 1.

Group A. Since 10 μg. of thiamin was found sufficient as a curative dose for even the most marked cases of polyneuritis associated with convulsions and at the same time was accompanied by very good growth, it was at first thought to use this daily amount as a standard of comparison. This was hardly justifiable, since the stock diet furnished in itself liberal amounts of vitamin B_1. On this daily dose fertility and lactation was perfectly normal for two generations. Therefore, it was considered advisable to use a large dose during the third generation. The daily dose was increased to 200 μg. thiamin daily. Toxic effects then became apparent. The lactation efficiency dropped from 95 to 41%; also, one female was sterile and post-mortem examination showed resorption of twelve remaining embryos. The resorption was indistinguishable from vitamin E deficiency.

Group B. No toxic effects were encountered in the first generation on the 100 μg. thiamin daily dose. In the second generation, however, two females were sterile, but the two females that were fertile reared their young successfully. In the third generation when the daily thiamin dose was in-

[3] We wish to express our appreciation to Merck and Co. for the thiamin donated for this work.

creased to 400 µg., five females completely failed to rear their seven litters and one was sterile. In other words, infant mortality was 100%. Almost invariably the litters died the first few days of the nursing period and canabalism was very marked, which was associated with a loss of the maternal instinct.

Group C. On the daily dose of 200 µg. thiamin, two mothers out of six became sterile in the first generation. Since only

TABLE 1

Influence of massive doses of vitamin B_1 on fertility and lactation

Group Vitamin B_1 daily dose	*Generation*	*Females*	*Litters*	*Young born*	*Young allowed to be reared*	*Young weaned*	*Per cent young weaned*	*Remarks*
A								
10 µg.	First	4	4	37	24	23	96	
10 µg.	Second	6	6	49	32	30	94	
200 µg.	Third	6	6	59	36	15	41	One female was sterile after 161 days of mating. Another female resorbed twelve embryos.
B								
100 µg.	First	4	4	36	24	24	100	
100 µg.	Second	4	2	21	12	12	100	Two females were sterile.
400 µg.	Third	6	7	64	42	0	0	One female was sterile after 110 days of mating.
C								
200 µg.	First	6	4	43	24	24	100	Two females were sterile.
200 µg.	Second	2	2	12	12	12	100	
600 µg.	Third	6	2	25	12	0	0	Four females were sterile 120 days after mating. One female resorbed fourteen embryos.
D								
400 µg.	First	4	6	61	34	32	94	
400 µg.	Second	6	6	69	36	30	83	
800 µg.	Third	6	14	147	78	10	12	

two mothers were available for the second generation, the insufficient number of animals are not a criterion for the fertility and lactation performances. In the third generation on the 600 µg. daily thiamin dose, however, out of six females four were sterile, one with a clear case of resorption indistinguishable from vitamin E deficiency and the other two that were fertile entirely failed to rear their litters, death of the young having occurred during the first few days of lactation.

Group D. It is surprising that on the 400 µg. daily thiamin dose, fertility and lactation should have proceeded normally during the first two generations, but lactation was a pronounced failure during the third generation on the 800 µg. daily vitamin B_1 dose, the infant mortality being 88%.

Growth in all instances with the thiamin supplements was much greater than on the stock diet alone.

At no time throughout this study was the regular stock diet with its supplements of milk and lettuce changed, and all the daily thiamin administrations were given orally in solution in Petri dishes, which all the animals eagerly consumed. The mothers were tame at all times, although when by virtue of the large doses of thiamin they were unable to lactate, they became disinterested in the welfare of their young and devoured them, a phenomenon characteristic of the experimental rat when there is a deficiency of an essential factor in the diet.

While Perla[4] on his stock diet began to encounter toxic symptoms on lactation in the first generation, the injurious effects on lactation of massive doses of thiamin, supplementing our stock diet were not manifested until the third generation. The injurious effects on fertility, however, are apparent in the first generation. Beginning with a 400 µg. thiamin daily dose the physiological mechanism of milk secretion

[4] Perla's recent findings ('39) that a daily allowance of 2 mg. manganous chloride counteracts the toxicity of large doses of thiamin lactation is of considerable interest. It would link vitamin B_1 metabolism with manganese, as vitamin D is associated with calcium and phosphorus metabolism. Also the giving of manganese salts might be indicated in various types of neuritis or other diseases where large doses of this vitamin are prescribed.

collapses. Out of 132 young allowed to be reared on 400 to 800 μg. daily doses of thiamin, only ten young were reared. Since this work was completed, Perla ('39) in a preliminary report also cites progressive decrease in fertility on daily supplements of 30 I.U. of vitamin B_1.

The fact that resorption of the foetus during gestation was observed in vitamin A deficiency (Sure, '28) and in toxic doses of vitamin B_1, we can no longer consider such phenomenon as specific for vitamin E deficiency.

The question arises: What daily dose of thiamin did the stock diet with its supplements provide? Before such calculation can be made it is necessary to know the thiamin content of each of the constituents of this diet expressed as micrograms per gram. From the thiamin content of foodstuffs given by Williams and Spies in their monograph ('38) whole wheat, rolled oats and yellow corn contain 4.72, 3.00, and 2.5 μg. per gram respectively. No figures are given for linseed meal nor for commercial casein. But it is estimated that linseed meal may contain 3 μg. per gram and commercial casein 2.0 μg. per gram. The latter should be a liberal figure, since milk powder contains only 2.5 μg. per gram. Lettuce contains 0.2 μg. per gram fresh tissue, and whole milk 0.45 μg. per gram. From such figures it is estimated that 100 gm. of our stock diet will contain about 328 μg. thiamin. A growing animal eating 15 gm. of such feed daily will then receive approximately 50 μg. of thiamin. A lactating rat which is eating 25 to 30 gm. of feed and drinking 10 to 15 cc. of fresh cow's milk, will consume 100 to 110 μg. thiamin daily. Since sterility was encountered in the second generation on 100 μg. of thiamin, supplementing our stock diet, which in itself provided an equal dose of this vitamin, it appears that twice the daily intake of thiamin furnished by our stock ration interferes with reproduction. Since 2.5 μg. thiamin daily per rat will prevent polyneuritic symptoms and loss of weight, eighty times the maintenance dose of thiamin is toxic from the standpoint of reproduction and 120 times the maintenance dose definitely toxic for lactation. Furthermore, studies just

completed on lactation with purified diets (using crystalline thiamin, riboflavin, B_6, choline, nicotinic acid and W factor as components of the vitamin B complex) indicate the daily requirement of vitamin B_1 to be 120 µg. In other words, three times the requirements for lactation (as found in short time transfer experiments from stock diet to synthetic diets) are definitely toxic in the third generation animals fed our stock diet. Just what manganese may do to counteract such toxicity (Perla, '39) is being checked at this time. When it is considered that Bills ('30) found that vitamin D (activated ergosterol) administered to rats in doses 100 times greater than the minimum antiricketic level did not produce a toxic effect on growth and reproduction, and that 1000 times the overdosage was just perceptibly harmful, and that it took a 4000 times overdosage to be definitely injurious, the possible toxic effects of vitamin B_1 or thiamin appear quite serious. It is doubtful, however, since vitamin B_1 is quite deficient in the American diet (Sure, '33; McCollum, '35) whether we will encounter injurious effects from an overdosage. However, we must bear in mind that large doses of thiamin for long periods of time may affect the pregnant and nursing mother.

SUMMARY

A daily dose of 100 µg. of thiamin results in female sterility in the second generation. A daily dose of 200 µg. of thiamin produces toxic effects in lactation in the third generation. A daily dose of 400 µg. of thiamin results in entire failure in lactation in the third generation.

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BASAL METABOLISM OF CONNECTICUT STATE COLLEGE STUDENTS

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THREE FIGURES

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A study of the basal metabolic rate of women in some of the nutrition classes at the Connecticut State College was started in 1933, but most of the work was done from 1935 to 1938. After 1933, the study was not confined to students in nutrition classes and included both men and women students.

Our original aim was to get some idea of the average basal rate of the students and to see how these averages compared with the averages of college students in other parts of the country. As work progressed the range in basal rates or the variations shown by individuals became a matter of considerable interest.

Basal metabolism studies by Hitchcock and Wardwell ('29) on college women have indicated that there is a cyclic variation in the basal metabolic rate. Studies by Tilt ('30, '35), Mason and Benedict ('34) and McKitterick ('36) have indicated that climate and altitude are factors that may effect basal rate. The observations of Coons and Schiefelbusch ('32) on type and quantity of food, of Hackett ('31) on coffee drinking and of Dill, Edwards and Forbes ('34) on smoking indicate that these factors may have some effect on the basal rate. Henry ('30) considers that overactive states and nervous or emotional strain may produce effects on the basal rate.

The Connecticut State College is situated at Storrs, Connecticut, a village 640 feet above sea level. The temperature range for 1937 was 6° to 96°F. with an average of 49.09°.[1] The climate is considered as good as may be found in the southern New England hills. The winters are not very cold and the summers are fairly cool. The village is 8 miles from the nearest city so is as nearly rural as any eastern Connecticut village.

One hundred and ten college women and fifty college men, 17 to 27 years of age are included in this study. These were selected from a large group and were all healthy and normal in every respect as far as could be determined by college health records or in any other way. The studies were carried on from September to June each year except 1937–1938 when a few students in the summer session were given tests.

The tests were made with a Benedict-Roth respiration calorimeter, which was kept in good condition and tested as to accuracy several times each year.

The students came to the basal metabolism room before breakfast and rested 15 minutes to ½ hour, depending upon the exertion necessary in getting to the room. Most of the women students came from the same building and came soon after awakening and without fully dressing. The men came from dormitories within 5 minutes walk of the basal metabolism room. Every student was questioned as to whether he had slept well and had slept the full number of hours that were customary for him. This varied from 7 to 9 hours. If the sleeping period had been curtailed the test was not given. If something had been eaten just before going to bed or if the student had smoked just before coming in, the test was not given that day.

Few of the students drank much coffee so this was not considered a factor of great importance. Most of the students ate in the college dining hall and a separate study of food intake was made on a group of students to see if it might

[1] The temperature average 1932 to 1937 was 48.47°F.

have any bearing on our average rate.[2] The women were tested at any time during the intra-menstrual period.

Information concerning the activities of the students during the preceding day or days was recorded. The pulse and body temperature were always recorded, and after the spring

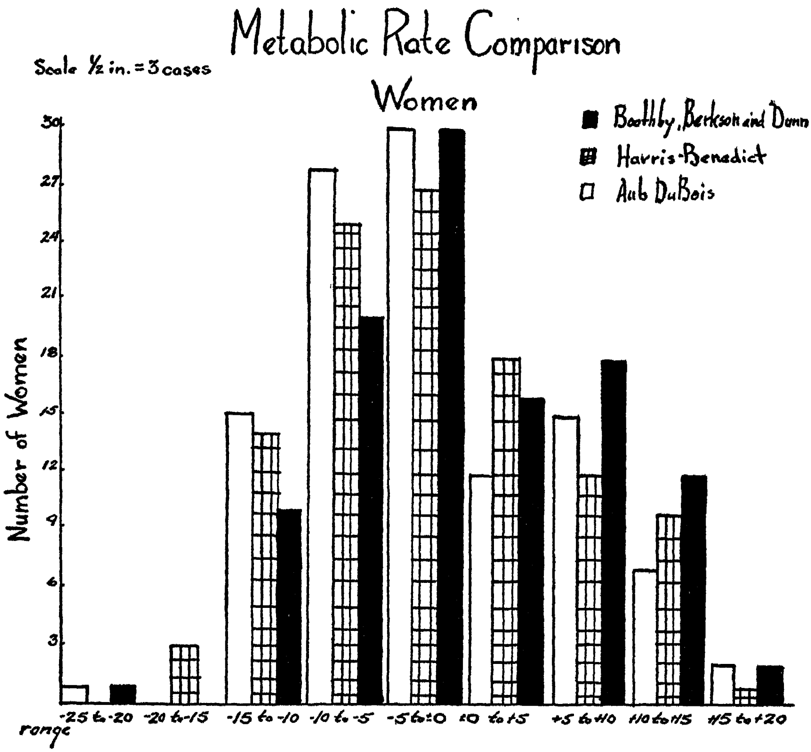

Fig. 1 Comparison of basal metabolic rates of women as calculated by the methods of: Boothby, Berkson and Dunn—Harris-Benedict—and Aub-DuBois.

of 1936 when a Baumanometer became available, blood pressure was determined.

During one winter the apparatus was taken to a room in the infirmary. The student to whom the test was given came in early, went to bed between 9 and 10 o'clock and was given the test at 7 o'clock the next morning without dressing

[2] Table not given in this paper.

or going further than the next room. As all students who came to the infirmary were carefully supervised it was possible to determine whether they were in true basal state.

A series of tests made in this way convinced us that they were in a true basal state and that our rest period in the basal metabolism room had been adequate. Variations in

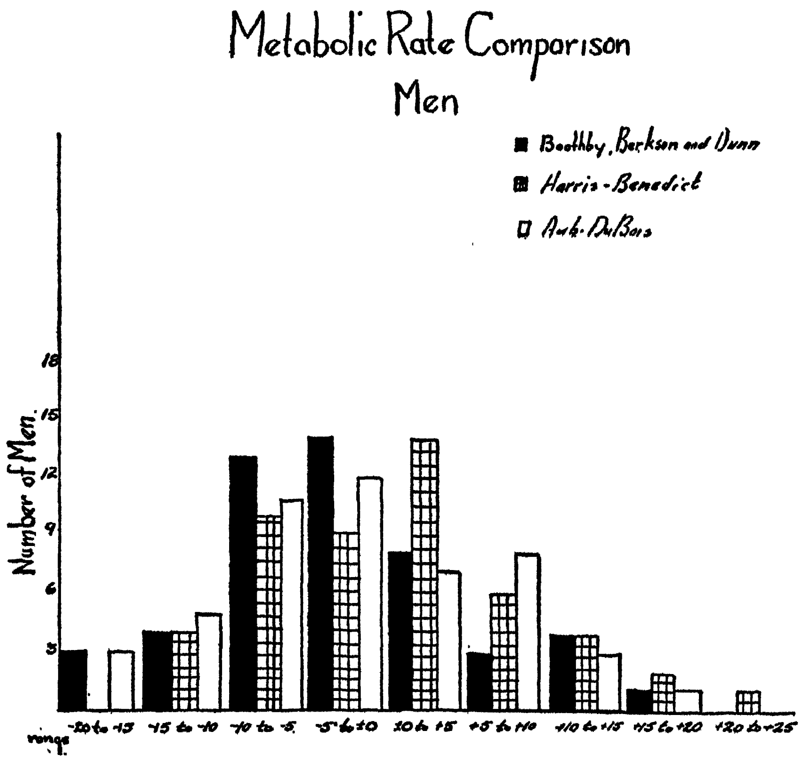

Fig. 2 Comparison of basal metabolic rates of men as calculated by the methods of: Boothby, Berkson and Dunn—Harris-Benedict—and Aub-DuBois.

basal rate of students given tests in both the infirmary and the dormitory basal metabolism rooms were insignificant. Day to day variations in basal rate when given in the same room were often greater.

Tests were given on 3 or more different days except in cases where tests on 2 different days were very nearly alike.

Early in our study two or more 6-minute tests were given each time the student came. It was seldom that there was much variation in consecutive tests given on the same day, so that this practice was discontinued except for a preliminary test the first time that a student came.

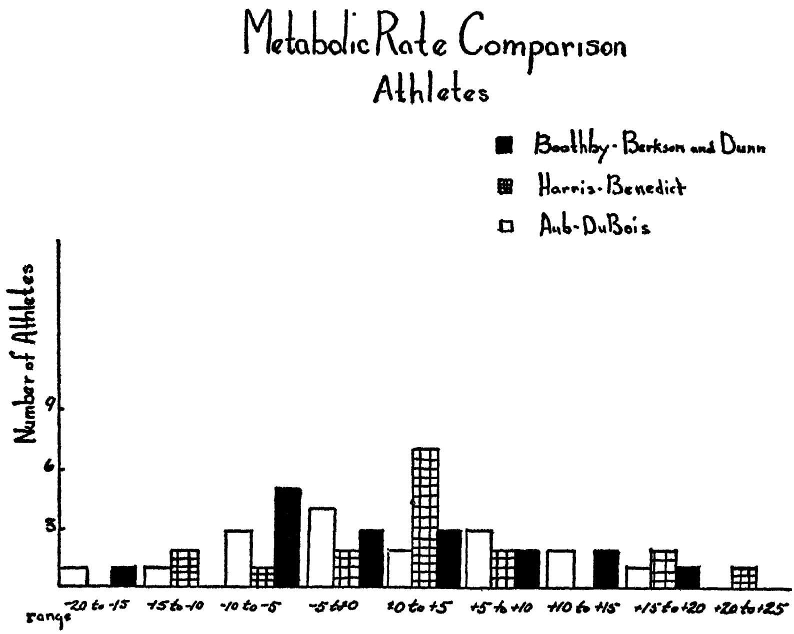

Fig. 3 Comparison of basal metabolic rates of athletes as calculated by the methods of: Boothby, Berkson and Dunn—Harris-Benedict—and Aub-DuBois.

RESULTS

The basal rates were calculated by the methods of 1) Boothby, Berkson and Dunn '36), 2) Harris-Benedict, and 3) Aub-DuBois, modified by Boothby and Sandiford.

Figures 1 and 2 are comparison graphs showing the differences in basal rate as calculated by the three above-mentioned methods. Figure 3 is that of athletes.

In spite of the very wide variation in range of basal rate the average rate for women is —0.4 by Boothby, Berkson and Dunn method; —1.9 Harris-Benedict, and —2.3 Aub-Dubois,

with probable errors of ±0.47 to ±0.52. The corresponding averages for men were —2.5, +0.8 and —1.6 respectively, with probable errors of ±0.74 to ±0.78.

DISCUSSION

The averages that were obtained in this study are nearer ±0 than some that have been reported for college students. It will be noted that the averages for women by the Boothby, Berkson and Dunn method —0.4 is a little higher than the averages —2.3 and —1.9 as calculated by the Aub-DuBois and the Harris-Benedict methods. The Aub-DuBois —2.3 coincides exactly with the Aub-DuBois figure reported by Gustafson and Benedict ('28) for a select group of Wellesley College students. Our averages are also higher than the figure —3.1 reported by McKetterick ('36) for Wyoming women where altitude is given as a factor increasing the rate. The figures —13.2 given by Coons and Schiefelbusch ('32) for Oklahoma women or —10.6 and —14.1 given by Tilt in two studies of Florida women ('30, '35) are considerably lower than our averages. In these cases quality and quantity of food as well as climate were considered factors that lowered the rate. Our averages for men —2.5 Boothby, Berkson and Dunn, +1.6 Aub-DuBois and +0.8 Harris-Benedict are a little higher than the —5.6 figure given by Nalbandov, Heller, Krause and Purdy ('38) in a study of Oklahoma college men.

In the case of the men students the average rate as calculated by the Boothby, Berkson and Dunn method is slightly lower than the Aub-DuBois and the Harris-Benedict methods.

In general it would appear that the Boothby, Berkson and Dunn method gives more normal curves for basal rates. The irregularities on curves for seventeen athletes are so similar to those for the larger group of fifty men that it seems probable that the athletes are responsible for these irregularities. Two groups of men as large as the group of women would probably be more satisfactory although the probable error in average rates for men is only ±0.74 to ±0.78.

The great length of range in basal rate of students who were considered normal in every way seemed to call for some explanation. Since we had carefully excluded any who were considered abnormal one might question whether students showing a wide range of basal rate were in true basal state during the tests. Those who showed the greatest range in basal rate usually averaged well within the ± 10.0. Usually the student whose rate seemed much too low or too high was unconscious of any emotional disturbance and seemed to us to be in true basal state.

W.B. a freshman gave a high rate of +18 at first but gradually it diminished to +4.1. At this time we found out that the swimming season had ended and that he was getting less exercise than usual. A.C. on the other hand gave such uniformity on two tests that the third seemed unnecessary. One test came on a day after a ball game and the other in a non-active period. All who knew him said that he was unusually uniform in studies and athletic activities.

Among the women, J.W. appeared to be in true basal state, but gave three plus rates, the highest of +28.7. She always had a normal pulse and blood pressure, and when we sent her to the college physician, he found her as on all other examinations, normal. After a vacation period of 10 days in which she slept and ate well the physician gave her a basal metabolism test. We also gave another test 2 days later. Both of these tests were near ± 0. She finally admitted that up until the vacation period she had been working hard and that perhaps had thought considerable about her studies and social activities. She said that she was not conscious of worrying but perhaps felt a bit let down when examinations and sorority elections were over.

J.L. was a student who was given the tests during a summer session when he was doing hard physical work in the college dairy department. His muscles were well developed and his slightly high rate was probably due to the effect of over-exercise.

Somewhat older people, members of the faculty and faculty wives who came to us for tests, rarely showed a great variation in basal rate. Often the variation in a series of three to five tests over a period of several weeks or months was only a few per cent. It seemed probable that the more settled or normal manner of living may account for the more uniform basal rate of this older group.

G. W. Henry ('30) says that elated and overactive states correlate with the high basal rate and that depressed states correlate with low rates. Henry suggests the probability that some of the normal variation in basal rate is due to the emotional state of the individual tested. It seems probable that the wide range or variation in basal rate of college students together with overactivity in some cases may be explained on this basis.

The average weight of the students tested was relatively high. Forty-five to 50% of both men and women are within 5% of the normal range.[3] The food intake seemed adequate in calories, vitamins and minerals, and the protein was 15%, or it was 4% higher than that reported by Coons and Schiefelbusch ('32) for Oklahoma women.

SUMMARY

The average basal rate of the Connecticut State College women was found to be —2.3 Aub-DuBois; —1.9 Harris-Benedict, and —0.4 Boothby, Berkson and Dunn. The average for men students was —1.6 Aub-DuBois; +0.8 Harris-Benedict, and —2.5 for Boothby, Berkson and Dunn. For an individual, ranges in rate may be of as much or more physiological significance than average rate.

The excellent quality and quantity of food may be a factor in producing a relatively high average, and the extremes of range may correlate with overactivity or with emotional states common to college life.

[3] From the records of Dr. Ralph L. Gilman, resident physician Connecticut State College, Storrs, Connecticut.

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THE CALCIUM CONTENT OF WHITE BREAD

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Published analyses of the calcium content of white bread do not give a true picture of the average amount of calcium in modern commercial bread. The data that have been available apparently are the result of investigations made many years ago. During the past few years, however, bakers have been incorporating relatively large quantities of milk solids in white bread, and they often add small amounts of calcium salts to the dough to compensate for deficiencies in local water supplies and to act as yeast nutrients or stimulators and as dough conditioners (hereafter referred to as yeast food). These practices have tended to increase considerably the average calcium content of white bread. In order to ascertain the present quantity of calcium in commercially produced bread, analyses have been made of thirty-nine representative commercial samples and eleven control samples.

Fairbanks ('38) conducted feeding experiments on rats and demonstrated that the addition of milk solids to a water bread (no milk) formula increases the nutritive value of bread. He points out that there is evidence that the nutritive value of bread containing 12% [1] milk solids is of higher order than bread containing 6% milk solids.

Frank and Wang ('25 a) report the calcium content of water bread as 0.029%,[2] Waller ('37) reports 0.027% and Rose

[1] All percentages in this paper referring to ingredients added to bread are on the basis of flour as 100%.

[2] Frank and Wang reported their results in terms of calcium oxide. The above results have been recalculated in terms of per cent calcium.

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('37) reports 0.029%. Rose ('20) gives the calcium content of white bread as 0.032%; Bridges ('35) and Sherman ('37) give 0.031%; and Fiene and Blumenthal ('38) give 0.027%. Neither Rose, Bridges, Sherman, nor Fiene and Blumenthal specify the bread other than as 'white bread.' Frank and Wang ('25 a) report 0.042%[2] calcium for bread containing milk (quantity of milk not given). Rose ('37) reports 0.064% Ca for bread made with milk. Although it was not reported, it is assumed that the results of the above authors are on an 'as received' basis (probably around 34% to 36% moisture).

TABLE 1

Per cent calcium in white bread as reported by various investigators

Per cent calcium 'as received basis' in

WATER BREAD	BREAD (INGREDIENTS NOT SPECIFIED)	BREAD CONTAINING MILK	INVESTIGATOR(S)
0.029	....	....	Frank and Wang ('25 a)
0.027	....	....	Waller ('37)
0.029	....	....	Rose ('37)
....	0.032	....	Rose ('20)
....	0.031	....	Bridges ('35)
....	0.031	....	Sherman ('37)
....	0.027	....	Fiene and Blumenthal ('38)
....	....	0.042	Frank and Wang ('25 a)
....	....	0.050 (38% moisture)	Sullivan and Howe ('29)
....	....	0.064	Rose ('37)

Sullivan and Howe ('29) report 0.080% calcium (dry basis) for white bread having 5.4% condensed milk and 0.235% yeast food. This would be 0.050% calcium on a 38% moisture basis. These data are summarized in table 1.

Morison and co-workers ('25) gave the following results, per cent calcium (table 2) for experimental loaves, the ingredients being weighed in grams, for varying amounts of dry whole milk. No yeast food was added to these loaves.

It was thought desirable to analyze for calcium a somewhat similar series of commercial control loaves (the ingredients being weighed in pounds and ounces), which con-

tained varying amounts of dry whole milk and dry skim milk and 0.25% yeast food. This was thought advisable in order to approach more closely the average commercial formula.

In addition to this, a request was made to bakeries throughout the country for loaves of their most popular white bread and also the percentage and kind of milk and yeast food, if any, used. The object of this request was to enable the authors to determine the average percentage of calcium in white bread available on the open market in the United States.

TABLE 2

Calcium content expressed in per cent. Analyses made on white bread containing various percentages of dry whole milk on basis of flour as 100% (Morison)

	NO MILK SOLIDS	2.5% MILK SOLIDS	4.0% MILK SOLIDS	5.5% MILK SOLIDS	7.0% MILK SOLIDS	8.0% MILK SOLIDS	9.0% MILK SOLIDS	10.0% MILK SOLIDS
Dry basis	0.016	0.045	0.059	0.069	0.090	0.097	0.111	0.119
38% moisture basis	0.010	0.028	0.037	0.043	0.056	0.060	0.069	0.074
(Calculated by Prouty and Cathcart)								

Loaves have been received and analyzed from six bakeries in Illinois, three bakeries in Louisiana, three bakeries in Tennessee, two bakeries in California, two bakeries in Washington, two bakeries in New York, and one bakery in each of the following states: Nebraska, Minnesota, North Dakota, Florida, Ohio, Maine, Massachusetts, Utah, Colorado, Rhode Island, Missouri, Iowa, Alabama, Kansas, South Carolina, Mississippi, Virginia, Pennsylvania, Montana and Washington, D. C. It is to be understood that these samples were the most popular types of white bread and were not necessarily loaves containing high percentages of milk solids.

EXPERIMENTAL METHOD

Type of ashing dish. A review of the literature failed to reveal the type of container most suited for ashing bread. Consequently, before attempting any work on calcicm analysis,

it was first necessary to determine the type of dish best suited for ashing. Forty separate ash determinations were made in duplicate. Determinations were run in porcelain crucibles and dishes, in silica crucibles and dishes and in platinum crucibles and dishes. The same sample was used throughout. The use of platinum did not meet with favor because it gave a hydrochloric acid solution of the ash a greenish color. The ash also stained the platinum to some extent. With all materials, the per cent ash obtained by the use of shallow dishes was slightly lower than that obtained by the use of crucibles, although both dishes and crucibles gave consistent results. Dishes were not chosen for the work because they were not as convenient to handle as crucibles. Porcelain and silica crucibles checked each other consistently.

Porcelain crucibles (top diameter 53 mm., height 44 mm., capacity 45 cc.) were selected because they were very easy to work with, gave as consistent results as silica, and were also much more economical than silica. On all the above samples, the ashing was done in an electric muffle at 550° C. for 12 hours.

Moisture and ash. The sample was prepared for analysis by the method described in Cereal Laboratory Methods of the A.A.C.C., page 83 ('35). Moisture and ash were run according to the official method of the A.O.A.C., which are described in the Cereal Laboratory Methods, pages 29–30. The vacuum method for moisture was used. For ashing purposes 10 gm. of air dried crumb were used.

Calcium. The analysis for calcium was performed by the Shohl and Pedley ('22) modification of the McCrudden ('09, '10, '11, '12) method. As Shohl ('22 a) pointed out, the hydrogen-ion-concentration is an important factor in making a calcium determination. If the solution is more acid than pH 4.0, calcium oxalate is dissolved, and if it is less acid than pH 5.6, magnesium ammonium phosphate is precipitated. Shohl and Pedley's modification consisted in precipitation at a specific pH. The only modifications on Shohl and Pedley's method were to leave out digesting with ammonium persulfate,

which was not necessary in bread analysis, and to titrate with 0.02N $KMno_4$ instead of 0.05N $KMno_4$.

Blanks were run on all reagents used along with every determination. Although improbable, tests were run to determine if any calcium was dissolved from the crucible with the hydrochloric acid used for dissolving the ash. The tests were negative in every case.

RESULTS AND DISCUSSION

Using the facilities of the Institute, several of the students were engaged in baking the control loaves used in the first part of the analysis. Ten pounds of flour per batch for each dough and 0.25% yeast food were used. Doughs were made containing no milk, 2, 4, 6, 8, and 10% dry skim milk and another series containing 2, 4, 6, 8, and 10% dry whole milk. The results are reported in table 3. Calculations of results to dry basis and 38% moisture basis were made separately from results of analysis on air dry crumb.

The results in table 3, as was to be expected, show that the per cent ash and per cent calcium increase as the per cent milk increases. Dry skim milk when used on the same percentage basis as dry whole milk gives higher calcium values. It will be noted that in general with each kind of milk the per cent calcium increases in fairly uniform increments with the exception of samples 11 and 13 for dry whole milk. The only explanation of this discrepancy is that the bread samples were made on a small commercial scale and weighed in pounds and ounces. It is evident that large errors could be introduced by a slight inaccuracy of weighing in pounds and ounces a batch containing 10 pounds of flour.[3]

Although most of the results for dry whole milk are in fairly good agreement with those of Morison and co-workers, there are discrepancies. About the only explanation that can be offered is that Morison's breads were made with ingredients which perhaps had slightly different calcium con-

[3] All samples which did not fall in line were re-run in duplicate, but in all cases the results checked within 0.003% calcium.

TABLE 3

Per cent ash and calcium of control loaves made with various percentages of dry skim milk (D.S.M.) and dry whole milk (D.W.M.) on the basis of flour as 100%; 0.25% Arkady (yeast food) was used in each dough

SAMPLE NO.	PER CENT MILK BASED ON FLOUR AS 100%	TYPE OF MILK	PER CENT WATER IN ORIGINAL LOAF[1]	PER CENT WATER IN AIR DRY CRUMB	PER CENT ASH 38% MOISTURE		PER CENT ASH DRY BASIS		PER CENT CALCIUM 38% MOISTURE		PER CENT CALCIUM DRY BASIS	
					Duplicate	Average	Duplicate	Average	Duplicate	Average	Duplicate	Average
					1.75		2.81		0.025		0.040	
5	0	....	30.0	8.2	1.75	1.75	2.81	2.81	0.025	0.025	0.040	0.040
					2.06		3.32		0.038		0.062	
6	2	DSM	29.0	8.0	2.07	2.07	3.34	3.33	0.040	0.039	0.065	0.064
					2.07		3.34		0.051		0.083	
7	4	DSM	29.7	8.0	2.07	2.07	3.34	3.34	0.052	0.052	0.084	0.084
					2.14		3.45		0.062		0.100	
8	6	DSM	29.4	8.3	2.14	2.14	3.46	3.46	0.063	0.063	0.102	0.101
					2.29		3.71		0.080		0.130	
9	8	DSM	29.7	8.2	2.29	2.29	3.71	3.71	0.081	0.081	0.130	0.130
					2.31		3.74		0.099		0.160	
10	10	DSM	29.4	7.9	2.30	2.31	3.72	3.73	0.096	0.097	0.156	0.158
					2.20		3.56		0.040		0.065	
11	2	DWM	28.4	7.5	2.20	2.20	3.56	3.56	0.041	0.041	0.067	0.066
					2.10		3.39		0.041		0.067	
12	4	DWM	27.7	7.5	2.10	2.10	3.39	3.39	0.043	0.042	0.070	0.069
					2.64		4.25		0.046		0.074	
13	6	DWM	28.5	7.4	2.64	2.64	4.25	4.25	0.047	0.047	0.077	0.076
					2.21		3.57		0.060		0.097	
14	8	DWM	27.8	7.4	2.21	2.21	3.57	3.57	0.060	0.060	0.097	0.097
					2.47		3.98		0.074		0.121	
15	10	DWM	28.1	7.3	2.45	2.46	3.96	3.97	0.073	0.074	0.119	0.120

[1] Loaves were not wrapped and moisture was not determined until 24 hours after baking.

tents than those used by the authors and contained no yeast food. This undoubtedly accounts for the lower calcium content of Morison's water bread. Thus, any increase in the milk content of bread would have a greater effect on a corresponding increase in per cent calcium of bread made from Morison's formula than bread made from the formula used by the authors.

The results for water bread of Frank and Wang ('25 a), Rose ('37) and Waller ('37) agree with the result of 0.025% calcium on 38% moisture basis as reported in table 3. The per cent calcium reported by Rose ('20), Bridges ('35), Sherman ('37), and Fiene and Blumenthal ('38) for 'white bread' are only slightly higher.

The results of the commercial breads which have been analyzed are reported in table 4.

From table 4 the average per cent calcium for the thirty-nine loaves, representing twenty-seven states, was 0.128 on dry basis and 0.080 on 38% moisture basis. The lowest figure found was 0.048% on a 38% moisture basis and 0.076% calcium on dry basis. The highest figure was 0.145% on a 38% moisture basis and 0.235% calcium on dry basis. The average ash was 1.86% on a 38% moisture basis and 3.00% on a dry basis. It is interesting to point out that the average per cent calcium found for white bread (38% moisture basis) is much higher than 0.048% calcium (dry basis) for wheat, as reported by Sullivan ('33). In the same article Sullivan reports 0.016% calcium (dry basis) for patent flour which gives some idea of the large increase in calcium of average white bread over the patent flour from which it is made.

Due to the increased amount of milk being used today in the bake shop, and also to the use of yeast foods, the calcium content of commercial white bread on the market today is much higher than previously reported. In white bread today, the percentage of milk is not necessarily an indication of the percentage of calcium; in some cases the calcium content is higher than would be indicated from milk content alone because of the various types and percentages of yeast foods being used

and because some yeast foods contain relatively large quantities of calcium salts.

Sherman ('37) points out that the human adult needs on the average a minimum of 0.45 gm. of calcium per day. Assuming that an average person consumes six slices of average white

TABLE 4

Per cent ash and per cent calcium of commercial white bread as sold on the open market in the United States

NUMBER OF BRANDS ANALYZED	PER CENT MILK BASED ON FLOUR AS 100%	TYPE OF MILK	AVERAGE PER CENT MOISTURE IN LOAF 'AS RECEIVED'	AVERAGE PER CENT ASH 38% H_2O BASIS	AVERAGE PER CENT ASH DRY BASIS	AVERAGE PER CENT CALCIUM 38% H_2O BASIS	AVERAGE PER CENT CALCIUM DRY BASIS
1	2.0	D.S.M.	35.4	1.84	2.96	0.065	0.104
2	3.0	D.S.M.	34.1	1.83	2.96	0.066	0.106
1	3.5	D.S.M.	34.3	1.83	2.96	0.072	0.114
3	4.0	D.S.M.	34.5	1.88	3.03	0.070	0.112
4	5.0	D.S.M.	33.3	1.81	2.92	0.076	0.122
4	6.0	D.S.M.	34.9	1.83	2.95	0.090	0.145
2	10.0	D.S.M.	34.5	2.03	3.28	0.141	0.229
1	7.0	D.W.M.	31.4	2.01	3.24	0.083	0.134
1	4.25	S.C.M.	35.1	1.76	2.84	0.055	0.089
1	15.0	S.C.M.	34.9	1.96	3.16	0.055	0.089
1	8.0	C.M.	34.5	1.69	2.72	0.051	0.082
1	9.0	C.M.	33.0	1.76	2.84	0.070	0.112
1	11.7	C.M.	34.8	1.87	3.10	0.059	0.095
1	10.0	S.C.S.M.	35.0	1.78	2.87	0.053	0.085
1	13.4	S.C.S.M.	24.1	1.93	3.12	0.118	0.191
10	Not reported	....	34.2	1.88	3.03	0.075	0.120
3	5.0	P.M.	34.1	1.83	2.95	0.087	0.142
1	{ 4.0 5.0	P.M. D.S.M.	34.0	1.96	3.16	0.114	0.184

DSM represents dry skim milk; DWM, dry whole milk; SCM, sweetened condensed milk; CM, condensed milk; SCSM, sweetened condensed skim milk; and PM, powdered milk (further details on this sample were not obtained from the baker).

bread per day, i.e., two slices at each meal, and that each slice weighs 1 ounce (28.35 gm.), which it does on the average, then, on the basis of the results obtained in this paper, this person will receive 0.136 gm. of calcium per day from bread.

This is 30% of his total calcium requirement for that day, assuming, of course, that all this calcium is assimilable.

Sherman ('37) and Fiene and Blumenthal ('38) report liquid whole milk as containing 0.118% to 0.120% calcium. Assuming one glass of milk is ½ pint, it will then contain on an average 0.285 gm. of calcium. Variable amounts of calcium may be obtained in vegetables and other foods. However, one glass of milk and six slices of bread a day (two slices at each meal) will furnish approximately an adult's total daily calcium requirement.

SUMMARY

Ten control loaves with varying amounts of dry skim milk and dry whole milk were analyzed to determine the effect of milk solids on the calcium content of white bread. As the percentage of milk solids was increased, the percentage of ash and of calcium in the loaf also increased.

Thirty-nine of the most popular commercial loaves of white bread from thirty-three bakeries and twenty-seven states have been analyzed for calcium. The average percentage of calcium found is nearly three times that reported by previous investigators; even the lowest per cent found is more than that previously reported and is greater than that reported by Sullivan for whole wheat berry. The increased use of milk solids and yeast foods has considerably increased the calcium content of commercial white bread.

On the basis of the results, six slices of average commercial white bread will supply approximately 30% of the daily calcicm requirement of the average adult, assuming the total amount of calcium is assimilable.

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SOME HISTOPATHOLOGIC OBSERVATIONS ON CHICKS DEFICIENT IN THE CHICK ANTI-DERMATITIS FACTOR OR PANTO-THENIC ACID [1]

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FOUR FIGURES

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It has been frequently observed that chicks on a ration low in the chick antidermatitis factor or pantothenic acid, developed neuromalacia. Generally the paralysis did not reach the typical 'curled toe' stage, but a slight paralysis was apparent. This paralysis was largely prevented by the administration of crystalline riboflavin.

Previous work ('38) has shown that riboflavin prevented neuromalacia of the peripheral nerves and that it would not completely prevent neuropathology in the spinal cord. Preliminary observations indicated that antidermatitis factor concentrates largely prevented the structural changes of the cord in chick dermatitis. It seemed desirable to determine whether one or both of these factors were involved in the nervous symptoms found in chick dermatitis. To this end a number of chicks suffering from dermatitis were examined from time to time. It soon became evident that systematic studies of this disease were necessary. With this in mind a general survey of the histopathology has been made and is herewith reported.

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EXPERIMENTAL

All birds studied in these experiments were day-old White Leghorn chicks fed diet 241H. This diet is a heated diet used by Mickelsen and his associates ('38). It produces marked dermatitis and death in the growing chick if unsupplemented by the antidermatitis factor. The first experiment[3] consisted of five lots of birds as follows:

Lot I—Basal ration 241H only.
Lot II—Basal ration + antidermatitis factor concentrate = 40% liver.
Lot III—Basal ration + riboflavin (4 μg. per gram of ration).
Lot IV—Basal ration + riboflavin (same as above) + B_1 + nicotinic acid.
Lot V—Basal ration + antidermatitis factor (same as in I) + riboflavin (same as in III).
Lot VI—Basal ration + 10 parts blackstrap molasses.

From five to ten birds were microscopically examined from each of these lots. A second experiment was likewise available. This experiment was composed of five lots of four birds each. One was fed 241H only, two lots were fed 241H plus 1% and 2% liver respectively for positive control and the remaining lots were fed the synthetic peptide made by coupling the acid chloride fraction of the alkali inactivated liver concentrate with the β-alanine ester as described by Woolley et al. ('39). Some of the birds reported by them were used for histologic study by us.

The sciatic nerve, spinal cord, liver and kidney were routinely examined with a few observations on the thymus, thyroid, striated muscle and skin.

The usual histologic technics were employed throughout these studies. The sciatic nerves were studied by means of the polarizing microscope, the Marchi reaction and by silver impregnation of axis cylinders. The spinal cords were studied for Nissl material by Einarson's method ('32), by the Marchi reaction and by the method of Bodian ('36). All other tissues were studied with the aid of hematoxylin and eosin.

[3] We are indebted to Mr. Harry A. Waisman for his cooperation in rearing the birds used in these experiments.

RESULTS

The observations made in these studies are summarized in table 1. It is seen that the spinal cord structures were affected in 100% of the cases unless concentrates of pantothenic acid were supplied. If the pantothenic acid concentrate was given without riboflavin neuromalacia developed in all cases. When the procedure was reversed and riboflavin

TABLE 1

This shows the number of chicks which developed histopathologic changes in the various organs and tissues that were studied

RATION FED	NUMBER OF CASES	NERVOUS SYSTEM		OTHER TISSUES				
		Sciatic	Spinal cord	Liver	Thymus	Skin	Muscle	Thyroid
241H only	14	5	14	1	2	12	1	..
241H + a.f.conc.[1]	10	10	0	..	1	0	1	0
241H + riboflavin	8	0	8	4	2	8	0	1
241H + a.f.conc. + riboflavin	5	0	0	2	0	0	0	0
241H + B_1 + riboflavin + nicotinic acid	7	0	7	0	2	..	..	..
241H + 10% molasses	3	0	0	0	..	..	..	..
241H + synthesis	6	3	0	0	..	..	..	..
241H + 1% liver	2	0	1	..	0	0	..	..
241H + 2% liver	4	4	1	0	0	0	0	0

[1] Antidermatitis factor concentrate.

alone was added, no changes in the sciatic nerve were observed. Here, however, degeneration in the spinal cord was present in all cases. If both a pantothenic acid concentrate and riboflavin were added, no changes were evident in either the spinal cord or the sciatic nerve. Nicotinic acid was ineffective in preventing the cord changes. The feeding of the pantothenic acid concentrate, made by the coupling of the acid chloride part of the alkali-inactivated liver concentrate with β-alanine ester, was effective in preventing cord changes, but the chicks developed histologic signs of neuromalacia. Liver at 1 and 2% was partially effective but was somewhat variable.

In most cases other tissues were less regularly affected. Occasionally the livers showed hydropic and fatty degeneration. If extra amounts of thiamin were added to the diet riboflavin was not effective in preventing these fatty livers. In not a few cases the thymus was in an advanced state of involution. This seemed to occur in those birds lacking in either pantothenic acid or riboflavin. It did not occur when both were present.

All cases of sciatic neuropathology were typical neuromalacia cases with myelin swelling, degeneration, clubbing and fragmentation of axis cylinders and Schwam cell proliferation as previously described ('38).

Lesions in the spinal cord were characterized by the myelin degeneration of myelinated fibers distributed widely throughout the white matter, except in the posterior region (figs. 1 to 4). Such degenerating fibers were observed to occur in all segments of the cord down to the lumbar region. In the brachial segment nerve cells show chromatolysis of the Nissl material. Such cells often presented an abnormal distribution of the Nissl which seemed to be an early stage of the more complete loss of the Nissl. These cases did not show a pronounced gliosis. It was noticed that in silver preparations vacuolar spaces were often observed in the cord cross section. In longitudinal sections degenerative changes were frequently observed in the axis cylinders of the affected nerves. In severe cases the nerve cells show fibrillae fragmentation.

Observations on the skin showed a dry sloughing of the keratinized epithelium. However, incrustations about the corners of the mouth and the areas between the toes usually showed distinct characteristics of dermatitis.

These studies support the preliminary observations published earlier with respect to the effect of the antidermatitis factor upon the neuropathology of the spinal cord ('38). It appears that pantothenic acid is definitely established as a constituent of and necessary to the maintenance of the normal intact elements of spinal cord.

It is seen that 10% molasses (blackstrap) protected against cord lesions and is indicative of its pantothenic acid content. Many times we have experienced increased severity of neuromalacia pathology when a ration low in riboflavin and pantothenic acid was supplemented by molasses. In these studies however there were no signs of sciatic pathology.

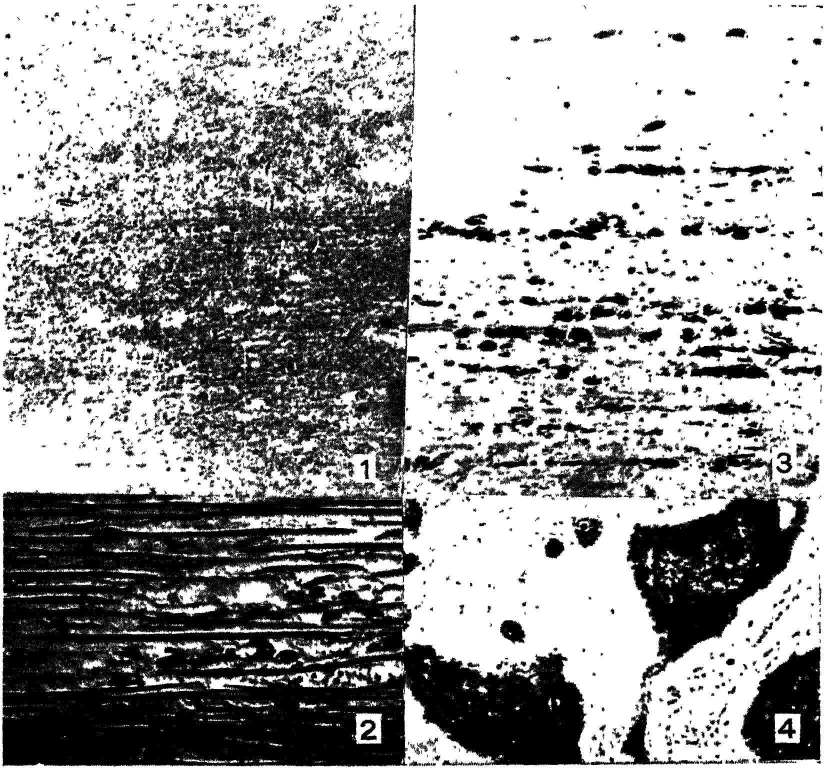

Fig. 1 Marchi preparation of the spinal cord of a chick fed ration 241H + pantothenic acid concentrate + riboflavin. × 400.

Fig. 2 Marchi section of the spinal cord of a chick fed ration 241H + thiamin + nicotinic acid + riboflavin. × 400.

Fig. 3 Bodian silver preparation of the spinal cord of a chick fed ration 241H only. Note the fragmentation of axis cylinders near the center of the field. × 400.

Fig. 4 Gallocyanin preparation from a chick fed ration 241H only. Chromatolysis and early Nissl changes probably due to lack of riboflavin. × 900.

No pathologic changes were observed in the brains of these chicks although the degenerating fibers high in the spinal cord would lead one to suspect some neuropathology in the higher centers. In these studies we have followed only the gross histopathologic aspects. The more minute histologic detail must await further study.

These studies show that the addition of pantothenic acid and riboflavin are necessary to eliminate neuropathology in chicks fed ration 241H. The former is necessary for the intact spinal cord and the latter for the peripheral nerves.

SUMMARY AND CONCLUSIONS

These data indicate that pantothenic acid is necessary for the maintenance of the normal intact structures of the spinal cord. Nicotinic acid, riboflavin or vitamin B_1 were not effective in preventing the spinal cord pathology in chicks fed ration 241H, while molasses proved to be effective. Chicks suffering from dermatitis often showed thymus involution, liver damage and keratitis and dermatitis in the skin. Fatty livers frequently occurred and were not prevented by riboflavin when thiamin was also fed. The synthetic pantothenic acid made from the β-alanine ester and the acid chloride portion of the alkali-inactivated liver concentrate were highly effective in eliminating the spinal cord pathology.

Pantothenic acid is necessary for the prevention of the neuropathology of the spinal cord in chicks suffering from dermatitis.

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OXALIC ACID IN FOODS AND ITS BEHAVIOR AND FATE IN THE DIET

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THREE FIGURES

(Received for publication April 25, 1939)

One is apt to gather from nutritional literature that vegetables do not supply calcium efficiently. Because this is not in accord with the results of a number of experiments, some covering several generations of animals (Kohman, Eddy and associates, '31, '34), this discrepancy presented itself as a problem wanting solution. As there seemed to be confusion about the amount of oxalates in foods and a scarcity of data as to the behavior and fate of oxalates in the diet, information on these points might be expected to throw light on the availability of calcium. It seemed desirable for higher accuracy, to make oxalate determinations under conditions that would avoid drastic treatment of the food and entail the concentration of the oxalate of a relatively large sample, freed from most of the other food constituents.

METHOD OF ANALYSIS

The following procedure was used. The samples, 400 gm., were first cooked tender by steaming without drainage loss. After thorough disintegration and an approximation of water content, sufficient concentrated hydrochloric acid was added to yield a 15% solution in the water present. This was

[1] The experimental work embodied in this paper was done in 1934–1936 while the author was with the Research Laboratory of the National Canner's Association, Washington, D. C. There he had the assistance of N. H. Sanborn and D. C. Smith. This opportunity is taken to acknowledge their part in the work.

allowed to stand at room temperature, with occasional stirring, not less than 2 days.

The sample was then pressed in cheese cloth and the weights of the drained material and of the residue recorded. By direct titration of a highly diluted sample, after filtration if necessary, the chlorine in the two fractions was determined. On the assumption that the oxalic acid distributed itself in the same ratio as the chlorine, it could be calculated for the entire sample by a determination on the drained fraction. This assumption was proved correct for pineapples and turnip greens by adding varying amounts of oxalic acid and subsequent analysis.

A 250 to 300 cc. sample of the drained liquid was subjected to 24 hours of wet ether extraction in an apparatus modeled after that suggested by Palkin, Murray and Watkins ('25) and suitably proportioned for this purpose using sintered glass to disperse the ether. In this the liquid to be extracted was 32 to 37 cm. deep and the ether coursed up through it from the 2.6 sq.cm. of fritted glass at a rate of 20 cc. per minute. When the extraction was complete, water was added to the extract, the ether evaporated and the water solution filtered. Calcium oxalate was then precipitated. After washing and redissolving a second, and if necessary, a third precipitation was made for complete purification. The precipitate was then titrated with permanganate. By precipitating the calcium in the titrated medium as calcium oxalate, the purity of the original oxalate could be gauged. All determinations were made in duplicate and the average is given in table 1. To study varietal and other factors more than one sample of some products were analyzed. As no significance could be placed on the differences, all the results were averaged and the number of samples is given in parenthesis.

THE OCCURRENCE OF OXALATES IN FOODS

Only a few foods, notably spinach, Swiss chard, New Zealand spinach, beet tops, lamb's quarter, poke, purslane and rhubarb have a high oxalate content. In them, expressed

TABLE 1

Oxalic acid (anhydrous) in foods

Product	*Total solids*	*Oxalic acid*	*Calcium*
Vegetables	%	%	%
Asparagus	6.05	0.0052	0.0201
Beans, green pod (3)	9.58	0.0310	0.0440
Beans, wax	7.80	0.041	0.054
Beans, lima (2)	24.25	0.0043	0.044
Beets, unpeeled	8.23	0.138	0.018
Beet leaves	6.60	0.916	0.120
Beet stems	6.66	0.338	0.040
Broccoli, leaves and flowers	10.50	0.0054	0.21
Broccoli, stalks	7.57	0.0035	0.092
Cabbage (2)	8.80	0.0077	0.189
Cabbage sprouts	8.52	0.0059	0.150
Cabbage, Chinese	6.45	0.0073	0.210
Carrots	11.02	0.033	0.044
Cauliflower	8.90	None	0.034
Celery stalks, bleached	4.58	0.034	0.054
Celery, soup leaves	14.66	0.050	0.55
Celery, soup stems	10.20	0.062	0.18
Collards (2)	12.75	0.0091	0.361
Chard, Swiss, leaves	9.47	0.66	0.11
Chard, Swiss, stalks	7.10	0.29	0.045
Chard, Swiss, leaves and stalks (2)	8.28	0.645	0.129
Chenepodium (Lamb's quarter)	8.20	1.11	0.099
Corn, sweet, white	25.00	0.0014	0.0076
Corn, sweet, yellow (2)	33.51	0.0052	0.0033
Cress, land, wild	15.00	None	0.24
Cress, early fine curled	8.80	0.0106	0.182
Cucumbers	3.72	None	0.014
Dandelions (3)	11.38	0.0246	0.171
Egg plant	6.18	0.0069	0.010
Endive (5)	7.58	0.0273	0.105
Escarole	6.10	0.0116	0.087
Kale	11.05	0.013	0.31
Kale, minus leaf ribs	18.05	0.011	0.294
Lettuce (6)	6.46	0.0071	0.073
Mustard greens (3)	8.40	0.0077	0.235
Okra	13.20	0.048	0.077
Onions, green	13.65	0.023	0.057
Parsley	13.70	0.19	0.29
Parsnips	22.70	0.010	0.049
Peas	19.50	None	0.019
Peppers, sweet, green (3)	7.34	0.016	0.0135
Poke	7.74	0.476	0.052
Potatoes, Irish	20.38	0.0057	0.0094
Potatoes, sweet	33.60	0.056	0.034
Purslane, leaves	9.45	0.910	0.13
Purslane, stalks	8.44	0.518	0.067
Radishes	3.75	None	0.028

TABLE 1—*Continued*

Product	*Total solids*	*Oxalic acid*	*Calcium*
Vegetables	%	%	%
Rape	10.82	0.0015	0.11
Rhubarb	6.62	0.50	0.044
Spinach (53)	10.35	0.892	0.122
Spinach, canned (12)	7.15	0.364	0.058
Spinach, New Zealand, leaves	7.60	0.89	0.11
Spinach, New Zealand, stalks	8.26	0.65	0.083
Squash, green summer	5.51	None	0.036
Turnips, peeled	8.16	None	0.037
Turnips, unpeeled	6.58	0.0018	0.028
Turnip greens	8.25	0.0146	0.245
Fruits			
Apples, early summer	12.58	None	0.010
Apricots	13.62	0.014	0.024
Avacados	14.60	None	0.0095
Bananas	23.81	0.0064	0.0071
Berries, black	12.25	0.018	0.038
Berries, blue	20.81	0.015	0.026
Berries, black rasp	22.10	0.053	0.058
Berries, dew	13.70	0.014	0.027
Berries, green goose	13.05	0.088	0.023
Berries, red rasp	14.10	0.015	0.023
Berries, straw	10.48	0.019	0.031
Cherries, red sour	12.18	0.0011	0.010
Cherries, sweet, Bing	24.50	None	0.0019
Currants, red	15.52	0.019	0.030
Grapes, Concord	15.30	0.025	0.024
Grapes, Thompson's seedless	23.90	None	0.013
Grapefruit	11.50	None	0.015
Lemons, juice	9.20	None	0.011
Lemons, peel	18.90	0.083	0.17
Limes, juice	10.39	None	0.015
Limes, peel	31.00	0.11	0.26
Mangoes	15.35	None	0.015
Melons, cantaloup	8.46	None	0.0090
Melons, casaba	11.22	None	0.0054
Melons, honey dew	6.08	None	0.0090
Melons, water	10.42	None	0.0060
Nectarines	14.45	None	0.0084
Oranges, edible portion	15.15	0.024	0.038
Oranges, peel	22.90	0.078	0.15
Peaches, Alberta	15.68	0.0050	0.012
Peaches, Hiley	14.10	None	0.0089
Pears, Bartlett	17.60	0.0030	0.014
Pineapples, Hawaiian canned	17.44	0.0063	0.019
Plums, damson	11.70	0.010	0.015
Plums, green gage	13.20	None	0.0080
Prunes, Italian	15.76	0.0058	0.12
Tomatoes	5.76	0.0075	0.010

as anhydrous oxalic acid, it is often considerably over 10% on a dry basis. One per cent in table 1 is equivalent to 1.4% of the usual crystalline form of oxalic acid. In most foods there are present mere traces. It is notable, however, that oxalate was obtained from all but a very few products, mostly fruits.

One purpose of the analytical survey was to ascertain if some varieties of spinach might be relatively free of oxalate. This hope was not realized. In fifty-three samples, including practically all commercial and many experimental varieties grown in California and in Maryland as well as those shipped from Texas, Florida and Carolina, the average anhydrous oxalic acid content was 9.02% on the dry basis (maximum 12.6, minimum 4.5). The calcium values averaged 1.25% (maximum 2.50, minimum 0.44). California spinach was only slightly lower in oxalic acid but markedly lower in calcium. California spinach averaged 0.59% calcium (maximum 0.84, minimum 0.44) while Maryland spinach averaged 1.92% (maximum 2.50, minimum 1.42). Since a considerable number of mineral elements are dietary essentials, such a variation raises a number of important questions.

PLAN OF FEEDING EXPERIMENTS

As a number of widely used greens contain insignificant amounts of oxalates, a comparison of these with spinach seemed in order. Green leaf-vegetables are our richest calcium sources. In previous experiments Kohman, Eddy and White ('37) used diets of canned foods and found them to supply calcium efficiently. For convenience, therefore, a basal diet of one can each of roast beef, peas, carrots and sweet potatoes was chosen. The essential analysis expressed in grams was:

One can	*Net weight*	*Solids*	*Calcium*	*Phosphorus*
Roast beef	362	133	0.054	0.155
Peas	607	111	0.106	0.474
Carrots	599	45	0.147	0.156
Sweet potatoes	551	184	0.133	0.251

By securing a sufficient amount from one lot of each of these, it was possible to have a constant, uniform supply for

an entire series of experiments conducted over an extended period of time. These four foods, in the above proportion, were thoroughly mashed and mixed, liquid and all, and hence had to be eaten in that proportion. This diet was obviously low in calcium, i.e., 0.093%. It permitted good but not maximum growth and bone formation, evincing efficient calcium. Small additions of greens (5 to 8%) were made to supply 60% of the calcium of the final mixture. This raised the calcium content to 0.22%.

In experiment 3, table 2, some additions to the basal diet were on a different basis. The turnip greens in diet C supplied only 58.8% of the calcium. It was added in that amount to be compared with diet D, in which it was accompanied by an equal amount of spinach, which was half the amount of spinach added in diet B to supply 60% of the calcium. The two greens in diet D supplied 70% of the calcium and raised the content to 0.3%. Also in experiment 3, diet D was planned to be equal to diets B and E on the basis of crude fiber, since the effect of crude fiber on calcium availability has often been questioned. Diet E is equal to D both in fiber and calcium.

The rats used were purchased from a large dealer who was able to supply large litters suitable for apportioning between the diets of a given experiment equally as to weight and sex. They were started on the experiments at 21 or 22 days of age when they weighed 30 to 35 gm. Six animals were always placed on each diet and kept in one cage with a raised wire bottom, three mesh to the inch. Except as noted otherwise the duration of the experiments was 21 days. There was an occasional death during this period and in every case it was an animal receiving either spinach or calcium oxalate.

RESULTS OF FEEDING EXPERIMENTS

It is apparent from data in tables 2, 3 and 4 that whereas spinach greatly increases the calcium content of the low calcium but well performing basal diet, it decidedly interferes with both growth and bone formation. This cannot be ex-

TABLE 2

Growth of rats and bone ash of alcohol-ether extracted dry tibia

Dietary addition		*Weight gain*	*Ash in tibia*
Experiment 1—21 days			
		gm.	*%*
Turnip greens (4.5%)		59	43.2
Spinach (8.2%)		38	33.4
Experiment 2—28 days			
None		67	44.7
Calcium carbonate		94	50.0
Calcium oxalate		79	47.6
Turnip greens (4.5%)		106	51.6
Spinach (8.2%)		77	45.2
Experiment 3—21 days			
None	Diet A	54	44.1
Spinach (8.8%–23.24 gm.)	Diet B	40	37.8
Turnip greens (4.4%–11.62 gm.)	Diet C	87	53.1
Turnip greens 11.62 gm. + spinach 11.62 gm.	Diet D	74	47.9
Spinach 23.24 gm. + $CaCO_3$ 0.406 gm.	Diet E	46	39.5
Experiment 4—21 days			
None		53	45.3
Canned spinach brand 1		47	40.3
Canned spinach brand 2		58	40.9
Canned spinach brand 3		57	43.5
Freshly cooked spinach		62	41.1
Experiment 6—21 days			
Calcium carbonate		76	51.1
Spinach, + $CaCO_3$ equivalent to oxalate in spinach		88	53.4
Spinach (7%)		57	40.5
Kale (6%)		79	53.5
Mustard greens (5%)		84	53.2
Experiment 7—21 days			
None		52	47.3
Canned spinach brand 1—not drained		45	45.7
Canned spinach brand 1—drained		54	45.1
Canned spinach brand 3—not drained		52	44.8
Canned spinach brand 3—drained		56	46.0
Freshly cooked spinach—not drained (9%)		54	45.5
Freshly cooked spinach—drained (7%)		75	49.1

plained on the basis of crude fiber. On the other hand greens with negligible oxalate content, such as turnip greens, kale, mustard greens and collards, markedly improve growth and bone formation under similar conditions. If enough calcium carbonate is added with the spinach to balance stoichiometrically its oxalate, performance in the rat is then comparable with other greens. This would require, in the fifty-three samples analyzed, from two to nine times as much calcium as the spinach contains. A general impression was obtained that some superiority, such as sleekness of fur, resulted when a low oxalate bearing green supplied calcium as against an equivalent addition of calcium carbonate.

In table 2 are recorded the results of a series of experiments in which the gain in weight and the per cent ash in the tibia was determined after feeding the basal diet alone and also the basal diet with various additions, to similar groups of animals. Each figure represents the average of six animals.

Table 3 records the gain in weight and the gain in calcium per animal in other similar feeding experiments. To arrive at the calcium per animal at the time the feeding was started, a similar group of six animals was sacrificed and their calcium content determined. In all cases when an entire animal was analyzed, the food was removed the evening before, i.e. 16 hours. In experiment 8, table 3, the food consumption was determined and from this the calcium utilization obtained. It appears that when turnip greens were added to the basal diet 79% of the calcium was utilized but when spinach was added only 15% was utilized. Assuming that the calcium supplied by the basal diet and the turnip greens was equally available, and bearing in mind that 15% of the total calcium is only 38% of that supplied by the basal diet, it appears that the spinach not only supplied no available calcium, but it actually rendered unavailable 41% of the calcium of the basal diet, i.e., the difference between 79 and 38. The sample of spinach used had 10.1% oxalic acid and 1.53% calcium. The diet to which it was added then had 0.8% oxalic acid. When calcium oxalate was the addition, 44% of the calcium was

utilized, showing that the rat can make use of a small portion of the calcium in it. It is notable that with turnip greens in the diet four times as much calcium per gram of tissue was deposited as with spinach in the diet. The data in tables 3 and 4, experiment 8, indicate a superiority of turnip greens over calcium carbonate in growth, calcium utilization and calcium deposition per unit body weight.

TABLE 3

Growth record and gain in calcium

Addition to basal diet	*Gain in weight*	*Calcium per rat*		*Calcium gain per gram gain in weight*	*Calcium utilized*
		Total	Net gain		
Experiment 5—21 days					
	gm.	*gm.*	*gm.*	*gm.*	%
Controls—21 days old	—	0.1853	—	—	—
Calcium oxalate	62	0.3535	0.1682	0.00267	—
Spinach (8.2%)	56	0.2449	0.0596	0.00106	—
Turnip greens (4.5%)	84	0.5300	0.3447	0.00410	—
Experiment 8—21 days					
Controls—21 days old	—	0.2172	—	—	—
Calcium carbonate	71	0.4205	0.2033	0.00286	70
Calcium oxalate	56	0.3286	0.1115	0.00199	44
Spinach	37	0.2447	0.0275	0.00074	15
Turnip greens	81	0.4603	0.2431	0.00300	79

RECOVERY OF CALCIUM AND OXALIC ACID IN URINE AND FECES

In a number of these experiments the calcium and oxalate excreted in the urine and feces were determined in addition to the calcium and oxalate in the food consumed and the calcium deposited in the tissue. This included any endogenous oxalic acid which would be overshadowed by the spinach oxalate. The data are recorded in table 4. The collection period was 7 or 8 days duration, thus allowing two periods in a 21-day experiment. The collection was begun 3 or 4 days after the animals were placed on a diet. There seems to be a tendency for less oxalic acid recovery in the second period, indicating that the ability to oxidize it increases with age. Also there is better utilization of calcium in the second period.

While urinary calcium may not seem high with spinach feeding, it represents a relatively high percentage of assimilated calcium, probably passing as oxalate through the excretory organs.

In only one case with spinach in the diet was as much of the calcium utilized as was supplied by the basal diet. The ex-

TABLE 4

Recovery of oxalic acid and calcium

Dietary addition		*Per cent oxalic acid recovered*			*Per cent calcium recovered*			*Calcium utilized*
		In urine	In feces	Total	In urine	In feces	Total	
		Experiment 2						
								%
Calcium oxalate		8	39	47				
Spinach		10	23	33				
		Experiment 3						
Spinach	Period 1	14	77	91				
	Period 2	18	72	90				
Spinach + $CaCO_3$	Period 1	12	84	96				
	Period 2	12	78	90				
		Experiment 5						
Spinach	Period 1	14	55	69				
	Period 2	10	34	44	10	60	70	30
Calcium oxalate	Period 1	6	47	53				
	Period 2	6	34	41	3	33	36	64
		Experiment 7						
Spinach not drained	Period 1	13	50	63	2	86	88	12
	Period 2	11	23	34	4	62	66	34
Spinach drained	Period 1	8	57	65	2	57	59	41
	Period 2	6	27	33	3	40	43	57
		Experiment 8						
Calcium carbonate	Period 1				18	8	26	74
	Period 2				9	6	15	85
Calcium oxalate	Period 1	13	55	68	3	43	46	54
	Period 2	6	73	79	3	63	66	34
Spinach	Period 1	11	33	44	3	73	76	24
	Period 2	8	24	32	8	62	70	30
Turnip greens	Period 1				10	8	18	82
	Period 2				3	6	9	91

ception is in table 4, experiment 7. In this the spinach was cooked by dropping it in twice its weight of boiling water, allowing 7 minutes to return to boiling and then boiling 10 minutes. After draining, its weight was 58% of the original. The undrained spinach was cooked 20 minutes in its adhering water. There was, however, very poor calcium utilization even with drained spinach. Numerous experimental and commercial blanching tests showed it is difficult to remove more than half the oxalate by this process. Spinach calcium is insoluble and hence not extractable.

EXTENDED FEEDING PERIOD

In one experiment twelve animals at 21 days of age were placed on each of two diets. In one group spinach supplied 60% of the calcium and in the other turnip greens. By the time the age of 90 days was reached, five animals on the spinach diet had died while all those on the diet containing turnip greens were in excellent condition. The average weight at 90 days of age of animals receiving spinach was 134 gm. while of those receiving turnip greens it was 205.

In due time two litters of nine young each appeared on the diet with turnip greens. These were reared to the age of 21 days when the average weight was 39 gm. in one litter and 34 in the other, while the average weight of their parents at the same age was 26 gm. Some time later one litter appeared among the animals on the diet with spinach, all but two of which were dead and these were shortly eaten by their mother.

Figures 1, 2 and 3 are photomicrographs of a tooth of an animal respectively on each of the three diets in experiment 3, table 2, i.e., the basal diet alone, with spinach added and with turnip greens. The band across which the line is drawn represents the dentine layer of a rat's tooth magnified 310 times. The dark portion of this band designated 'A' is calcified while the light portion 'B' is uncalcified and represents the area where new dentine is being formed. The wide uncalcified area in figure 2, representing the spinach diet is

apparent. It should be mentioned here that the bones of animals receiving spinach were very soft and pliable.

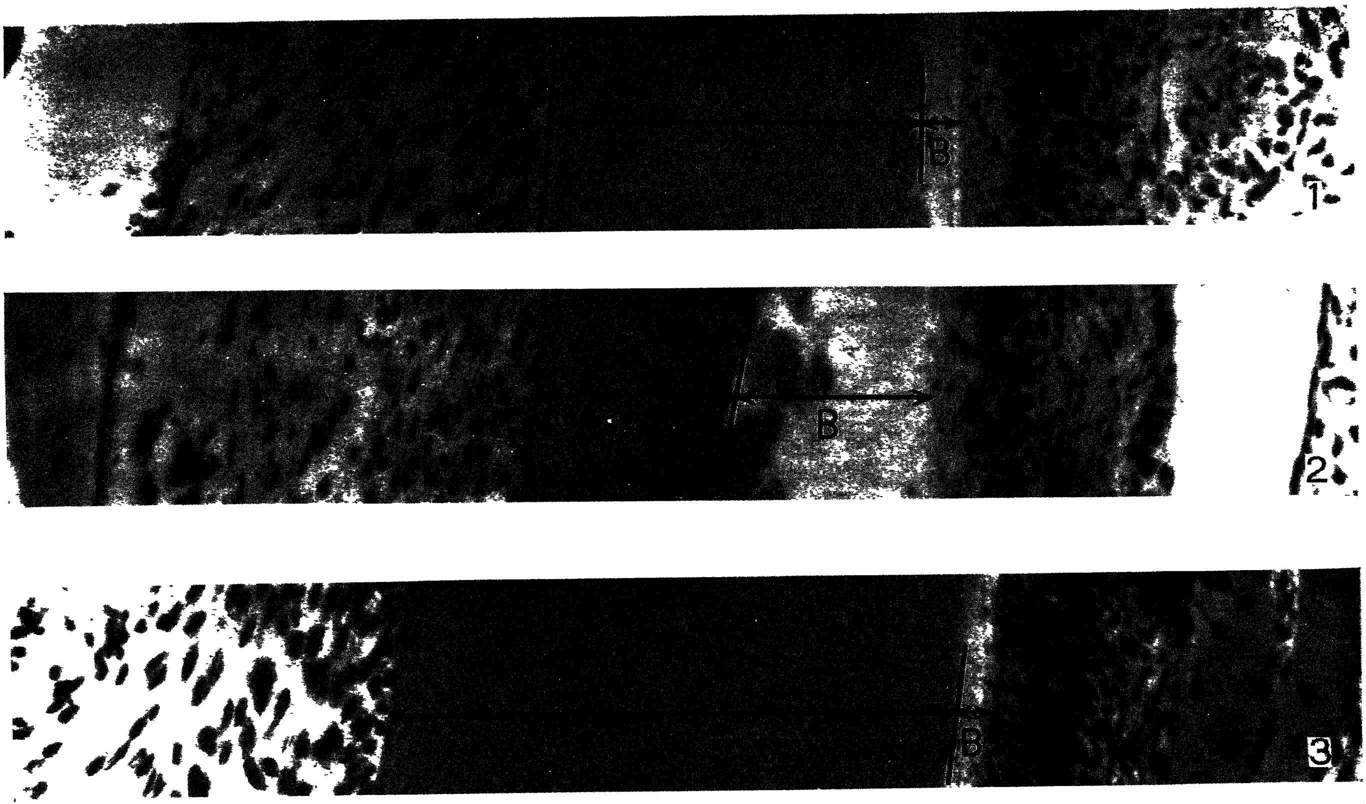
DISCUSSION

Insofar as these data are on a comparable basis they are in agreement with the reports of Tisdall and Drake ('38), Fairbanks and Mitchell ('38) and Fincke and Sherman ('35), published since this work was done. They all dealt with calcium availability. No one seems to have studied heretofore the fate of the oxalates in the diet of the rat. This is perhaps of equal importance. Some oxalic acid is assimilated and tends to carry calcium with it into the urine. In view of the low solubility of calcium oxalate, there arises the question of any possible damage of its passage through the excretory tissues. The demonstration of almost universal presence of small amounts of oxalates in vegetables and fruits tends to throw doubt on the quantitative aspects of endogenous oxalic acid.

CONCLUSION

Oxalates, expressed as anhydrous oxalic acid, have been shown to occur to the extent of about 10% on a dry basis in spinach, New Zealand spinach, Swiss chard, beet tops, lamb's quarter, poke, purslane and rhubarb. Traces were found in nearly all vegetables and fruits.

If to a diet of meat, peas, carrots and sweet potatoes, relatively low in calcium but permitting good though not maximum growth and bone formation, spinach is added to the extent of about 8% to supply 60% of the calcium, a high percentage of deaths occurs among rats fed between the age of 21 and 90 days. Reproduction is impossible. The bones are extremely low in calcium, tooth structure is disorganized and dentine poorly calcified. Spinach not only supplies no available calcium but renders unavailable considerable of that of the other foods. Considerable of the oxalate appears in the urine, much more in the feces.



Photomicrographs, × 310. Dentine of rat's tooth; 'A' calcified, 'B' uncalcified area. Figures 1, 2, and 3 respectively from animal receiving basal diet only, basal diet with spinach and basal diet with turnip greens, diets A, B and C respectively in experiment 3, table 2.

Turnip greens, mustard greens, kale and collards, greens with negligible oxalates, under similar conditions produce excellent animals that deposit four times as much calcium per unit body weight as those receiving spinach.

Acknowledgment is made to Capt. A. L. Irons, Dental Corps, U. S. A., who prepared the photomicrographs, and to the experiment stations at Davis, California, Beltsville, Maryland and Geneva, New York, where various samples were obtained.

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THE DISTRIBUTION OF THE CHICK ANTIDERMATITIS FACTOR (PANTOTHENIC ACID) IN MEATS AND MEAT PRODUCTS [1]

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The chick antidermatitis factor is now recognized as a specific member of the vitamin B complex and distinct from the antipellagra factor. Earlier work by Koehn and Elvehjem ('37) had established that purified liver fractions which prevented the typical dermatitis in chicks were also active in the cure of blacktongue in dogs. Later work by Elvehjem, Madden, Strong and Woolley ('38) showed that the anti-blacktongue activity of these liver fractions was due to the presence of nicotinamide. However, when nicotinic acid or nicotinamide was fed to chicks placed on the heated diet, it was found that these compounds were completely inactive in preventing the typical dermatitis (Mickelsen, Waisman and Elvehjem, '38; Dann and Subbarow, '38). Thus the chick antidermatitis factor is distinct from the antipellagra factor. Recently, in a communication from this laboratory (Woolley, Waisman and Elvehjem, '39), the partial synthesis of the chick antidermatitis factor and its relation to pantothenic acid has been reported. Jukes ('39) has also reported that pantothenic acid is the chick antidermatitis factor. No extensive investigations have been made on the distribution of pantothenic acid in animal tissues, and we wish to report here the distribution of the chick antidermatitis factor in meats and meat products.

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EXPERIMENTAL

The basal ration 241H used in these assays is composed of yellow corn 58, wheat middlings 25 and crude casein 12; $CaCo_3$ 1; $Ca_3(PO_4)_2$ 1; NaCl 1; hexane extract of alfalfa leaf meal equivalent to 1% of the original meal. The corn, middlings and casein were heated for 30 hours at 120°C. Adequate amounts of vitamin A and vitamin D were provided by biweekly doses with halibut liver oil. The basal ration was supplemented with 100 μg. of thiamin per 100 gm. of ration. Since the quantity of meat which replaced part of the basal furnished some vitamin B_1 itself as shown by work in this laboratory (Mickelsen, Waisman and Elvehjem, '39), the total amount of vitamin B_1 furnished the chicks was more than sufficient for their requirement (Arnold and Elvehjem, '38). In order to insure an adequate riboflavin intake in our rations aside from that furnished by the meat, 200 μg. of crystalline riboflavin (Merck) were added to each 100 gm. of ration. The rations were made up weekly and fed ad libitum.

Day-old White Leghorn chicks were secured from commercial hatcheries and from the Poultry Department of the University. The chicks were placed on raised screens in heated brooders and were weighed weekly. Observations were made daily for lesions on the beak and legs and around the eyes.

The tissues which were used in these assays were of two kinds. One set of samples was obtained from Chicago packers.[2] Samples 1 to 32 were ground vacuum dried, and vacuum packed in tin containers before shipment to us. The remainder of the samples was prepared in our laboratory according to the method of drying described in our earlier publication (Mickelsen, Waisman and Elvehjem, '39). In evaluating the potency of these tissues in the chick antidermatitis factor, we have determined the minimum level of the tissues required to prevent the appearance of lesions in all

[2] We wish to thank Wilson and Company, Armour and Company and Swift and Company for furnishing these samples.

chicks of the group over a 5-week assay period. Although Jukes and Lepkovsky ('36) have described a method for roughly calculating the concentration of the factor in the diet from the growth response, we feel that until a generally accepted standard is available, it is best to report the minimal level of the tissue needed for protection.

In order to determine these minimal levels, the tissues were fed at various percentages of the basal heated diet. It was found that levels of the tissue either higher or lower than the minimal protective level showed corresponding graded increments in growth and incidence of symptoms. Typical results

TABLE 1

Typical increments in growth of chicks fed increasing levels of tissue

TISSUE	SAMPLE NO.	LEVEL FED	AVERAGE WEIGHT AT 5 WEEKS	NUMBER OF BIRDS	NUMBER WITH SYMPTOMS	NUMBER DEAD AT 5 WEEKS
Pork liver	29	0.5	90	5	4	1
	29	1.0	122	4	3	0
	29	2.0	167	4	1	0
	29	4.0	223	8	0	0
Beef lung	78	10	95	4	2	2
	78	20	135	4	1	0
	6	30	172	4	0	0
	6	40	199	4	0	0

obtained with chicks fed these various levels are shown in table 1. When pork liver was fed at 0.5, 1.0, 2.0 and 4.0% levels, much better growth was obtained on the 4% level although complete protection from the typical dermatitis was secured at 2%. The average weight for the four surviving birds on the 0.5% level was 90 gm. at the end of 5 weeks, but all the chicks showed the typical symptoms. The average weight for the chicks on the 1.0% level was 122 gm., but only three out of four birds had the dermatitis. The chicks on the 2% level had an average weight of 167 gm. after 5 weeks and only one bird showed the syndrome. It was found that eight birds on the 4% level averaged 223 gm. and all were completely protected. Another indication of the better growth

obtained on levels of tissue which were higher than that needed for complete protection is that of beef lung. The average weight of the two surviving birds on the 10% level was 95 gm., and these showed the typical dermatitis. The birds on the 20% level averaged 135 gm. after 5 weeks, but only one out of four showed the symptoms. With 30 and 40% levels there was complete protection and growth was increased to 172 gm. and 199 gm. respectively.

Table 2 presents a short summary of the results of the assays for the chick antidermatitis factor (pantothenic acid). Approximately 2% of the liver from the different animals studied was sufficient to protect the chicks. Three samples of beef liver, three of lamb liver, and two samples of veal liver gave protection at the 2% level. The minimal protective level of pork liver must be set at 3% since one out of five birds on the 2% level had the symptoms. It should be pointed out that although 2% of the liver is borderline on the basis of the minimal protective level, the average weight of the birds in the group receiving the 2% pork liver was more than twice that of the basal group at 5 weeks. Pork kidney was active at 1%, while 2% of beef kidney was necessary for complete protection. Lamb kidney was not fed below 5%. Beef spleen was active at 15% while beef brain gave complete protection at 5%. The minimal protective level for beef heart is 5% since all the chicks in the group at this level were entirely free from symptoms, while three out of four chicks on the 3% level exhibited the dermatitis. One of the chicks in the group fed beef lung at a 20% level showed the dermatitis whereas a level of 30% furnished complete protection to the chicks. Two birds out of four in each of two groups receiving 5% and 7.5% respectively of beef pancreas showed the symptoms, thus placing the minimal protective level for pancreas at 10%. Beef tongue gave complete protection at 15%.

In the case of beef muscle there was an indication that the better grade of meat contained more of the chick antidermatitis factor than the meat of lower quality. A sample of sirloin butt (sample 18) from an animal graded at the time

TABLE 2

Summary of the assays for the chick antidermatitis factor

TISSUE	SAMPLE NO.	COMPLETE PROTECTION AT LEVELS	LOWEST LEVEL FED	MINIMAL PROTECTIVE LEVEL
		%	%	%
Beef brain	11	20,30	20	
	57	10,20	10	
	77	5	5	5
Beef heart	13	10,20	10	
	53	10,20	10	
	73	5,10	3	5
Beef kidney	9	3,5	3	
	49	3,5	3	
	84	2,3,5	1	2
Beef liver	10	2,4	2	
	58	2	1	
	98	2,4	1	2
Beef lung	6	30,40	30	
	78		10	25
Beef muscle	18		20	
	19	30	20	
	40		20	
	105	35	25	30
Beef spleen	4	20	10	
	76	20	10	15
Beef pancreas	79	10,20	5	10
Beef tongue	82	15	5	15
Lamb kidney	22	5,10	5	<5
Lamb liver	23	4,10	4	
	61	2,3	2	
	96	1,2	1	1
Lamb muscle	35	30	30	
	80		25	30
Pork kidney	28	3,5	3	
	62	2,4	2	
	83	2	1	1
Pork liver	29	4	0.5	
	86		1	3 or 4
Pork muscle	12	20,30	20	
	24	20,30	20	
	25		20	
	26		30	
	30		20	
	32		20	
	34		20	
	51	30	20	
	47	20,30	20	20–30
Veal muscle	16	40	40	
	44	25,35	25	
	75		25	25–35
Veal liver	5	4	2	
	97	2	1	2
Stewed beef heart	87	7.5,10	5	7.5
Stewed beef spleen	88		5	15
Stewed beef kidney	85	5	3	5
Fried beef liver	99	1,2,4		1

of slaughter as fair did not offer complete protection at a level of 20% whereas a similar cut from a prime steer (sample 19) did give protection at the same level. A third sample of prime beef (sample 40) also offered protection at a 20% level. Still another sample of beef (sample 105) gave protection at a level of 25%. One sample of lamb muscle (sample 80) did not offer protection at either a level of 25% or 30%, but 30% of sample 35 did give complete protection and good growth.

Nine different samples of pork muscle were assayed and in general it appears that the minimal protective level is between 20 and 30%. There was considerable variation in the potency of these samples. Pork loin (sample 30) did not protect the chicks from dermatitis nor did it produce good growth when fed at levels as high as 30%, whereas sample 24 at a level of 20% afforded protection and induced fair growth. A sample of pork shoulder (sample 25) and one of pork ham (sample 26) produced borderline protection at levels of 20 and 30% respectively as shown by the fact that one chick in each of these groups had slight symptoms of dermatitis. Another sample of pork ham (sample 51) did not give complete protection at the 20% level but did give it at the 30% level. Three samples of veal muscle also showed variations in their ability to prevent chick dermatitis. Sample 44 afforded complete protection at a level of 35% while sample 75 at the same level was not able to protect the birds completely. Sample 16 at a level of 40% gave complete protection and good growth.

Although it had previously been shown that the chick anti-dermatitis factor was labile to dry heating but stable to autoclaving, no reports have appeared on the influence of cooking on this factor. A few cooked samples described in our earlier paper (Mickelsen, Waisman and Elvehjem, '39) and a sample of fried beef liver were assayed in an effort to determine the destruction produced by these methods of cooking. The stewed beef heart had to be fed at a level of 7.5% in order to produce complete protection while the uncooked beef heart was active at a level of 5%. Stewed beef kidney was protective at a level of 5% which is in contrast to the activity of the

uncooked sample at a level of 2%. The sample of stewed beef spleen was fed at a level of 15% in order to produce complete protection whereas the uncooked sample was active at a level of 10%. We have found that fried beef liver fed at a 1% level protects the chicks from dermatitis but does not allow good growth. When this sample was fed at a 2% level there was complete protection and good growth, indicating that under the method of frying employed, there was no destruction of the factor. It is of interest to note that we have observed slightly better growth on the 2% of fried beef liver than on 2% of the untreated tissue of the same animal. In one series the average weight of the birds receiving the untreated liver was 172 gm. at 5 weeks, while that of the birds receiving the fried liver was 263 gm. A comparison of the average weight of twelve chicks on 2% of untreated liver with the average weight of ten chicks on the fried liver gives 202 grams and 241 gm. respectively.

DISCUSSION

The results of these assays indicate that liver and kidney from various animals are two of the richest animal sources of the chick antidermatitis factor. A level of 2% will furnish complete protection and produce good growth. Beef heart and beef brain are next in potency with the minimal level at 5% while between 10 and 15% of beef spleen are required for protection. Beef pancreas is protective at 10% and beef tongue is active at the 15% level. In contrast to these values the muscles of pork, beef, lamb and veal are required at levels between 25 and 35% before any protection is obtained. Beef lung was also required at a level between 25 and 30% before it gave complete protection. Jukes and Lepkovsky ('36) found that 24% of meat scraps was a poor source of the filtrate factor. Jukes ('37) fed vacuum dried beef round at 25 and 50% levels and concluded that it had a low filtrate factor value. We have found that the minimal protective level for beef muscle is 25% and is of much lower potency when compared to the organs of the same specie.

The influence of stewing on the chick antidermatitis content of beef heart, beef spleen and beef kidney is readily brought out by a comparison of the untreated and cooked samples. It can be concluded that approximately one-third of the potency was lost by this method of cooking. This decreased potency of the cooked meats can be best explained by the partial extraction of the factor by the water in which the meat was cooked. The influence of frying on the potency of liver is evidently not of significance since the chicks were protected from symptoms at the 1 and 2% levels. At present it is difficult to explain the increased growth obtained in the groups receiving the fried liver over that of those receiving the uncooked tissue. This finding was consistent in a number of trials and cannot be attributed to a fortuitous choice of chicks.

It is interesting to consider the variations in the amount of the various water soluble vitamins in the same tissue. A comparison of the distribution of the chick antidermatitis factor with that of vitamin B_1 indicates that the highest concentration of vitamin B_1 was found in pork ham, yet this tissue was lowest in the chick antidermatis factor. The values for liver and kidney also demonstrate the wide differences in the occurrence of the two factors. Here again the liver and kidney are comparatively low in vitamin B_1 when compared to other tissues and are the richest in the chick antidermatitis factor. We found very little variation in the chick antidermatitis factor content of these tissues obtained from different species. The minimal protective level for pork, beef and veal liver was 2% while the muscle tissue from lamb, beef, pork and veal offered protection at levels between 25 and 30%.

Work from this laboratory (Peterson and Elvehjem, '39) indicates that from 2 to 6% of dried yeasts grown on various media will give complete protection from dermatitis in chicks fed the heated basal diet. Dried liver and kidney can be considered equally good sources of the chick antidermatitis factor (pantothenic acid) since these tissues are also active at the 2% level.

The use of our heated basal diet 241H was fully satisfactory for the assay of the chick antidermatitis factor (pantothenic acid). We have obtained growth with this diet when a good source of the chick factor was supplied. However, an interesting observation was made on the growth of the chicks receiving certain levels of the tissues. Fairly good growth was observed in some of the groups even though the chicks showed typical dermatitis. When 1% of beef liver was fed to one group, the four birds showed the syndrome but the average weight reached 115 gm. at 5 weeks which is nearly twice that of the chicks on the basal diet. In the case of 10% beef spleen (sample 76) the average growth was 154 gm. but three birds showed dermatitis. Typical lesions were also observed in three of the chicks getting 5% beef tongue (sample 82) while the average growth was 117 gm. These few examples are sufficient to suggest a possible distinction between the chick antidermatitis factor and a growth-promoting substance. We cannot say definitely at present that the factor which protects against dermatitis in chicks will also stimulate growth or that the two factors are distinct. While our work was in progress, Bauernfeind et al. ('38) indicated that a new factor was required for growth and reproduction in the domestic fowl. They found that the factor is destroyed by the method of preparation of the heated diet. Although the minimal protective levels of our tissues protected the chicks from symptoms and at the same time produced good growth, the lower levels of the same tissues frequently produced much better growth than that obtained on the basal ration even though the birds showed dermatitis. This is evidence that the heated diet is probably deficient in a factor other than that which protects against chick dermatitis.

SUMMARY

1. The minimal protective levels for the chick antidermatitis factor (pantothenic acid) in meat and meat products have been determined.

2. The liver and kidney of various species were found to be the richest sources of the factor, followed by heart, spleen, brain, pancreas, tongue and lung. The muscular tissues of beef, lamb, pork and veal had the least potency.

3. The ordinary household method of stewing decreased the potency of kidney, heart and spleen by one-third.

4. The factor in liver was not destroyed by frying. On the contrary, better growth was consistently obtained with the fried beef liver than with the uncooked tissue.

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ON THE APPROXIMATION OF THE CALCULATED TO DETERMINED CALCIUM CONTENT OF HUMAN DIETARIES

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It is a matter of some practical importance to determine how closely the composition of diets can be approximated by calculations based upon tables giving mean analytical values for each component foodstuff. This applies particularly to the calcium content of human dietaries since it is known that the coefficient of variation with respect to the calcium content of many food stuffs is conspicuously large (Sherman, '37). Where analyses of diets for calcium content have been made and the results compared with values computed by reference to various tables (Bauer and Aub, '27; Bassett and van Alstine, '35), the determined values have been found to be significantly and quite consistently lower than the calculated estimates. A discrepancy in the same direction and of similar degree was noted by us in the course of our own metabolic studies.

Sherman's continued investigations of the composition of foods have led to revision of the mean calcium content of certain foodstuffs (Sherman, '37). It seemed to us of interest to compare the determined with the calculated calcium content of a number of representative diets, utilizing the revised data recently made available. As the validity of such comparisons depends, in part, upon the variation inherent in our methods of analysis, we have summarized in table 1 our results of determinations made in consecutive periods of

representative diets. Table 1 indicates the range of variation in found values attributable to variations in the calcium content of raw foodstuffs plus technical errors in sampling, due to losses in cooking of foods and in chemical analysis. It should be emphasized that the determined values used for comparisons with Sherman's figures for raw foodstuffs (Sherman, '26, '37) are the results of analysis of cooked foods

TABLE 1

Results of calcium analyses of diets in consecutive 5-day periods showing scatter due to Σ variations in 1. Ca content of raw foodstuffs, 2. sampling, 3. losses in cooking, 4. chemical analyses

Diet	*Period*	*Determined Ca content of diet* (*mg. per day*)	*Deviation from mean* (*mg.*)	*Diet*	*Period*	*Determined Ca content of diet* (*mg. per day*)	*Deviation from mean* (*mg.*)
A	1	911	—39	E	1	473	+ 5
	2	945	— 5		2	460	— 8
	3	994	+44		3	468	± 0
		Mean = 950			4	448	—20
B	1	926	+55		5	494	+26
	2	848	—23		6	460	— 8
	3	892	+21		7	472	+ 4
	4	878	+ 7			Mean = 468	
	5	810	—61	F	1	466	+14
		Mean = 871			2	450	— 2
C	1	1072	—27		3	446	— 6
	2	1106	— 3		4	432	—20
	3	1240	+131		5	448	— 4
	4	1068	—41		6	468	+16
	5	1088	—21		7	456	+ 4
	6	1122	+13		8	452	± 0
	7	1066	—43			Mean = 452	
		Mean = 1109		G	1	678	— 2
D	1	465	+21		2	696	+16
	2	452	+ 8		3	674	— 6
	3	474	+30		4	690	+10
	4	438	— 6		5	746	+66
	5	410	—34		6	668	—12
	6	448	+ 4		7	660	—20
	7	422	—22		8	670	—10
		Mean = 444			9	668	—12
					10	656	—24
					11	678	— 2
						Mean = 680	

prepared under conditions designed to minimize leaching out of minerals.

METHODS

The general procedure outlined by Bauer and Aub ('27) was followed in our mineral balance studies, which were conducted in the metabolism wards of the Presbyterian Hospital under the supervision of a specially-trained personnel. The dietaries were calculated and the raw foods weighed and prepared in a diet kitchen adjoining the metabolism ward, under the direction of the dietitian in charge. In order to minimize variations in composition, quantities of raw food sufficient for one 5-day period (or two 3-day periods, in some instances) plus enough food for 1 extra day were purchased from uniform sources at the beginning of the metabolic period. Foods subject to quick deterioration were divided into two portions, the part not required for immediate use being stored in a special low-temperature refrigerator. In order to minimize loss of mineral content, the food was weighed, cooked and served in the same dish; in the case of vegetables and fruits (when not served raw) the cooking being done in high glazed, covered casseroles by pressure without addition of water. Meats were broiled on a large-mesh wire grill over a casserole to catch drippings and were served in that casserole. The extra day's aliquot of food was weighed and prepared exactly as for the trays tendered daily to the patient but was submitted to the laboratory for analysis. There the foods with their juices were removed quantitatively, minced and thoroughly mixed by hand at first, more recently with a meat grinder and mechanical stirrer. The food mass was dried, pulverized and triplicate 1 to 2 gm. samples removed for ashing in a muffle furnace. After treatment of the ash in the manner described by Tisdall and Kramer ('21), calcium was determined by the Clark and Collip ('25) modification of that method.

RESULTS

Table 2 summarizes comparisons between determined values for the calcium content of diets and values calculated by reference to tables published by Sherman in 1926 and to the revised 1937 figures. The data comprise twenty-eight observations made in the course of metabolic studies on twenty-one cases during the period 1930 to 1939. As the comparisons appear to vary significantly according to the dietary calcium level, they are divided into two groups: high and intermediate calcium diets containing more than 400 mg. per day, and low calcium diets containing less than 200 mg. per day. The data in each group are arranged in order of increasing negativity of the value determined minus calculated (by 1937 figures), expressed in percent of the calculated estimate. The month and year of each observation are recorded though no definite seasonal trend could be recognized.

The point of major interest in these results (table 2) concerns the consistency and degree of the positive deviation from determined values observed in calculated estimates, whether based upon the 1926 or upon the revised 1937 figures. The calculated estimates exceeded determined values more consistently in low calcium diets (in fourteen of sixteen observations) than in intermediate or high calcium diets (eight of twelve observations using the 1937 figures). In these latter diets, too, the scatter in percentage deviations of the calculated values was found to be smaller, showing a total range of +13 to −24% as compared with +16 to −49%. The absolute error in milligrams calcium, however, may be much larger in high than in low calcium diets.

The use of Sherman's revised figures for the mean calcium content of foodstuffs invariably yielded lower net values for total calcium content than were obtained with the use of earlier figures. The difference was small in low calcium diets where it was due almost entirely to lower mean values for orange and other fruit juices.

In general, the revised figures appear to afford better agreement between determined and calculated values for the cal-

TABLE 2

Comparison of determined with calculated values for the calcium content of human dietaries

Case	Date	Calcium in diet (mg. per day) Determined	Calculated (Sherman 1926)	Calculated (Sherman 1937)	Determined minus calculated (Sherman 1926) mg.	Determined minus calculated (Sherman 1937) mg.	Det. minus calculated / Calculated (S 1937) %
		I. High and intermediate calcium diets					
Mc.	III-31	923	840	803	+83	+120	+13
Oc.	IV-31	1109 [1]	1041	1001	+68	+108	+10
Wo.	XII-32	992	1006	941	−14	+51	+5
Wo.	XII-32	981	1006	941	−25	+40	+4
St.	I-37	680 [1]	715	688 [2]	−35	−8	−1
Mm.	V-37	1716	2589 [3]	1765		−49	−3
Pa.	XI-30	871 [1]	952	906	−81	−35	−4
Mk.	X-37	2010	2259	2105	−249	−95	−5
Fl.	X-36	452 [1]	541	507 [2]	−89	−55	−12
Bo.	II-38	444 [1]	566	530	−122	−86	−19
Mm.	V-37	1178	1560	1473	−382	−295	−20
Bo.	I-38	468 [1]	644	579	−176	−111	−24
		II. Low calcium diets					
No.	II-36	126	113	109	+13	+17	+16
Mo.	X-38	138	136	131	+2	+7	+5
Sc.	VII-38	157	180	168	−23	−11	−7
Gr.	VIII-33	118	131	128	−13	−10	−8
Li.	VIII-36	96	119	115	−23	−19	−17
Li.	VIII-36	94	119	115	−26	−21	−18
Mh.	XI-37	128	174	173	−46	−45	−26
De.	V-37	115	169	161	−54	−46	−29
Ba.	VI-37	126	210	183	−84	−57	−31
We.	IV-38	110	180	163	−70	−53	−32
Sa.	IV-33	80	131	127	−51	−47	−37
Mm.	IV-37	70	119	115	−49	−45	−39
Mm.	IV-37	69	119	115	−50	−46	−40
Wo.	XII-32	73	130	126	−57	−53	−41
Gu.	IV-38	100	183	171	−83	−71	−42
Le.	II-39	44	101	86	−56	−42	−49

[1] Mean of several periods (see table 1).

[2] Milk powder (Dryco) used instead of milk and calculated 10 gm. Dryco = 95 gm. skimmed milk.

[3] Cottage cheese calculated as hard cheese.

cium content of diets than do the older figures. This obtained, for example, in twenty-two of the twenty-eight diets in our series. However, even with the use of the revised figures the calculated estimates were still too high in twenty-two of the twenty-eight diets studied; in our sixteen low calcium diets the positive deviation exceeded 25% of the calculated value in ten instances. Loss of calcium in cooking may account for part of this discrepancy. But because of the precautions taken in this regard, we doubt that the discrepancy can be explained wholly in this way, particularly in the low calcium diets in which the major portion of the calcium content was supplied by uncooked foods.

Our figures emphasize again the hazards of employing calculated estimates of the calcium content of diets when conducting calcium balances for clinical or metabolic studies. Variables affecting the absorption and utilization of calcium from dietary sources constitute further sources of error not indicated by our analyses.

SUMMARY

The calcium content of sixteen low calcium diets and twelve intermediate and high calcium diets was determined by analysis. The results were compared with the dietary calcium content as computed by reference to tables giving mean calcium contents of the component foodstuffs. In twenty-two of twenty-eight observations, Sherman's revised ('37) tables gave better agreement between calculated and found values than did earlier figures. A disproportionate number of calculated estimates, however, whether based upon earlier or upon revised data, exceeded the determined values, the positive deviation being greater than 25% of the calculated value in ten of sixteen low calcium diets.

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THE RAT GROWTH FACTORS OF THE FILTRATE FRACTION OF LIVER EXTRACTS [1]

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ONE FIGURE

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The B group of vitamins has been shown to contain one or more unknown substances in addition to the factors thiamin, riboflavin and vitamin B_6, which already have been isolated (Schultz, '37, '38; Lepkovsky, '36; Frost, '37; Edgar, '37). Both yeast (Edgar, '37; Schultz, '37, '38) and liver extracts (Lepkovsky, '37; Oleson, '39; Frost, '39; Edgar, '38 a) have been used as sources of the unknown substances. The extracts from the two sources have been found to have similar, but not identical, properties. Specifically, Edgar and Macrae ('37) reported that treatment of yeast extracts with fuller's earth results in a sharp partition of the rat growth-promoting activity. When given singly, neither the filtrate nor the adsorbate has more than slight activity; but when both are given, complete growth results. Edgar, El Sadr and Macrae ('38 a) found that fuller's earth treatment did not yield inactive fractions when applied to liver extracts. However, fractions with the same biological properties as the yeast eluate and filtrate fractions could be prepared by other means.

On the other hand, Oleson, Bird, Elvehjem and Hart ('39) report that when their liver extracts are treated with fuller's earth, neither the filtrate nor the eluate actively promotes

[1] These investigations have been supported in part by the Ella Sachs Platz Foundation and the Proctor Fund of Harvard University.

growth. Moreover, when both fractions are given, the animals fail to grow normally, although symptoms of deficiency diseases are prevented.

The present paper is concerned with the study of the rat growth-promoting substances of the so-called '95% alcoholic filtrate' of liver (Cohn fraction F, '27). This starting material, on treatment with fuller's earth, yields two fractions, both of which are relatively inactive when given separately but in combination have the full activity of the original extract. The 95% alcoholic filtrate, therefore, is a convenient starting material for a study of the growth-promoting substances of the fuller's earth filtrate. A report of the progress made on this problem follows.

EXPERIMENTAL

Diet. The basal diet (BF) used consists of the following: casein 200, peanut oil 80, cod liver oil 20, sucrose 120, cornstarch 500, wood flour 40, salts (Osborne and Mendel, '19) 40. The casein is heated on the water bath under reflux condenser with 2.5 parts of 95% ethanol for a period of 6 hours; this procedure is repeated twice.

For most of the experiments reported herein, the basal diet was supplemented by the addition of fuller's earth adsorbate (S4). When used, 5.4 gm. of fuller's earth adsorbate, prepared as described below, was incorporated in 1 kg. of the basal diet.

Thiamin and riboflavin were given in doses of 70 and 80 γ, respectively, three times each week to all animals.

The various liver fractions under investigation were administered orally from a 1 cc. calibrated hypodermic syringe in equal doses (usually 0.2 cc.) three times each week.

Experimental animals. All the albino rats used for the experiment were from inbred stock of The Wistar Institute strain. The mothers and young were placed on the basal diet of the experiment (either S4 or BF) when the young reached a weight of 30 to 35 gm., and the young were weaned and isolated when they reached a weight of 40 to 50 gm. at about

24 days of age. Unless otherwise noted, each test group contained six animals, three males and three females, in most instances each pair coming from a different family.

Preparation of liver extract fractions

Commercial 95% alcoholic filtrate, a by-product in the preparation of the pernicious-anemia curative material in pig's liver extract, was used as the starting material for all supplements. Each cubic centimeter of this solution represents material from aproximately 100 gm. of raw liver. The concentration of each of the various fractions has been referred to the quantity of original liver represented in the starting material.

Fuller's earth filtrate and adsorbate. The 95% alcoholic filtrate was treated at pH = 1 with 0.5 gm. of fuller's earth for each 100 gm. of liver represented in the solution. After stirring for about 30 minutes, the solution was filtered and the adsorbate was washed with water and air-dried. This adsorbate was used without elution as the S4 supplement. An aliquot of the filtrate was diluted to a concentration equivalent to 60 gm. of liver per cubic centimeter. This has been designated as fraction S2.

The procedures used for concentrating the active substance of the fuller's earth filtrate are illustrated in chart 1.

Hydrolyzed preparations (S2H2, S1H3). To solutions of the ether precipitate (S22) and the purified fuller's earth filtrate (S13), respectively, H_2SO_4 was added until the solution was distinctly acid to thymol blue. H_2SO_4 in an amount equivalent to 0.2 normal additional acid in the final solution then was added. Finally, the solution was heated for 60 minutes on a boiling water bath, cooled, neutralized with NaOH and filtered.

Fraction S22 treated as described above is designated as S2H2, and fraction S13 as S1H3.

Mercury filtrate (S33) *and precipitate* (S30). Purified fuller's earth filtrate (S13) was treated with an excess of mercuric acetate (1.2 volumes of 20% mercuric acetate solu-

CHART 1

Steps in the concentration of the growth-promoting substances of liver extracts

Inactive fractions	*Partially active fractions*	*Active fractions*
		95% alcoholic filtrate. Extract with amyl alcohol at pH 4.5
Aqueous residue ←		
		Amyl alcoholic solution. Extract with NaOH
Amyl alcoholic solution ←		
		Aqueous solution. Treat with charcoal
Aqueous solution ←		
		Charcoal adsorbate. Elute with 60% aqueous ethanol
Charcoal residue ←		
		Alcoholic eluate. Evaporate. Dilute with water (S13). Neutralize to pH 7.0. Continuous ether extraction for 24 hours
Ether extract (S19) ←		
		Aqueous solution. Acidify with 1/15 volume of 10 N H_2SO_4. Continuous ether extraction for 48 hours
	Acid aqueous residue (S27) ←	
		Ether extract. Evaporate (S18). Neutralize with brucine. Dry. Dissolve in chloroform. Extract with water.
Chloroform solution ←		
		Aqueous extract. Convert to calcium salts. Concentrate. Add 10 volumes of absolute ethanol
	Precipitate (S23) ←	
		Alcoholic solution. Add ether in excess
	Ether filtrate (S25) ←	
		Precipitate (S22)

tion) and the mixture was allowed to stand overnight in the refrigerator. The mixture was centrifuged, and the precipitate was washed once with water. The precipitate and the combined filtrate and washings were decomposed in the cold with H_2S. The solutions were aerated, filtered and the filtrates were neutralized with sodium hydroxide solution.

RESULTS

The results obtained in testing the biological activity of the various fractions described in the preceding section are presented in table 1. The results have been expressed as the average gain in weight per week for each group (six rats except where otherwise stated). Most tests were run for at least 5 weeks. The average gain per week for a 5-week period has been tabulated in the last column and forms the basis for evaluating the relative activity of each fraction. In general, fractions were considered active when the rate of gain in weight exceeded that of the controls by 50%.

In order to illustrate the differences in rates of growth obtained with different fractions, examples of the experiments with fractions S4, S13 and S22 have been plotted in figure 1. The significance of the results will be discussed subsequently.

It should be noted that even with the addition of supplements which give excellent initial weight increases, the rate of gain is not maintained throughout the 5-week period. This suggests that even the crude fractions S2 and S4 do not contain all the substances essential for sustained growth in optimum amounts (v.i., p. 273).

The basal diet without added liver supplement supported a negligible increase in weight. Addition of the fuller's earth filtrate (S2) alone gave an average weight increase of 9.3 gm. per week and of the fuller's earth adsorbate (S4) alone gave an increase of 6.7 gm. per week. Administering the two together gave a weight increase of 18.4 gm. per week. Therefore, progress in concentrating the active material of the fuller's earth filtrate could be followed by measuring the

growth obtained upon administering the various fractions to animals receiving S4 in the diet.

Using these criteria it was found that the growth-promoting factors could be shaken out into amyl alcohol from acid solution, taken back into dilute sodium hydroxide, adsorbed on carbon and eluted with aqueous ethanol without great loss (S13). From this preparation part of the activity could be

TABLE 1

Biological response to various liver extract fractions added to basal diet BF

SUPLEMENT ADDED TO BASAL DIET			AVERAGE GAIN IN WEIGHT EACH WEEK					AVERAGE GAIN PER WEEK 5-WEEK PERIOD
Experiment no.	Description	Amount as liver equivalent in grams per week per rat [2]	1	2	3	4	5	
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
..	None	..	0.0	0.3	0.0	0.3	—0.5	0.0
S2	Fullers' earth filtrate	36	8.0	9.2	9.3	9.3	10.9	9.3
S4	Fullers' earth adsorbate	60–90	7.2	9.0	7.5	6.0	3.8	6.7
S2+S4	Fullers' earth filtrate	36	18.3	17.9	20.7	20.2	14.9	18.4
S13+S4	Purified F.E.F.	60	25.0	20.7	20.0	17.0	14.6	19.5
S19+S4	Neutral ether extract	140	4.8	6.0	7.0	8.0	8.0	6.8
S18+S4	Acid ether extract	140	20.0	16.5	15.0	..	..	(17.2)
S18+S4	Acid ether extract	690	22.5	25.5	17.0	12.2	..	(19.3)
S27+S4	Residue from ether extraction	140	8.2	10.8	15.0	11.5	11.3	11.4
S23+S4[1]	Alcohol precipitate	640	9.8	16.8	19.5	11.5	..	(14.4)
S22+S4[1]	Ether precipitate	320	20.5	15.8	17.3	11.3	9.8	14.9
S22+S27+S4[1]		[3]	16.3	18.7	22.2	11.7	12.8	16.3
S25+S4	Ether filtrate	580	9.5	16.5	14.5	12.3	..	(13.2)
S2H2+S4	S22 hydrolyzed	290	7.8	4.3	6.0	8.5	8.5	7.0
S1H3+S4	S13 hydrolyzed	60	10.5	14.7	13.7	12.9	11.9	12.7
S33+S4	Mercury filtrate	60	12.8	23.2	20.2	15.8	6.8	15.8
S30+S4	Mercury precipitate	60	11.3	10.0	6.8	7.7	..	(9.0)
S33+S30+S4		[3]	15.5	20.8	19.0	17.8	9.2	16.5
S19+S22+S4		[3]	14.7	19.0	20.2	11.8	11.0	15.3

[1] Four rats instead of six were in each of these series.

[2] This refers to the amount of supplement other than S4, which when supplied was constant throughout.

[3] Each supplement was administered in an amount equal to that shown for the same supplement when given above.

extracted by ether from acid (S18) but not from neutral (S19) solution. The procedure was attended by considerable loss indicated by the increase in the amount of liver equivalent needed to promote rat growth. Neither the extract (S18) nor the residue (S27) is as active as the original material (S13).

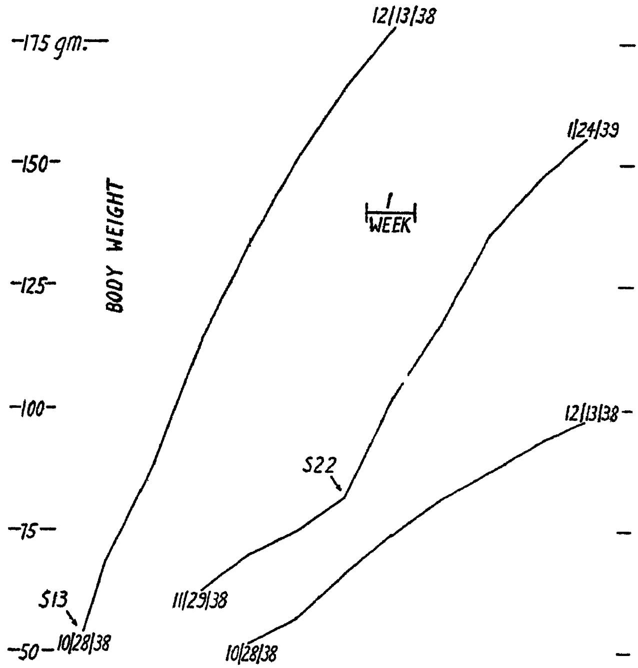

Fig. 1 Examples of average weight curves of rats given various liver extracts as supplements to the basal diet. Experiments with S13 + S4 and S4 were each carried out with a group of six rats; the experiment with S22 + S4, with a group of four rats. All animals had received the supplement S4 since weaning. The other supplements were added at the times indicated by the arrows.

Moreover, when the most active fraction of the ether-extractable material is given along with the residue (S22 + S27) the activity of the original extract is not reached even when both fractions are given in greater amounts. The ether extraction, however, serves the useful purpose of separating much of the active substance from a large amount of inert material.

When the brucine salts, from S18, are partitioned between water and chloroform, the greater part of the activity goes into the aqueous phase. This was shown by determining the activity contained in the calcium salts of the fractions S23, S22 and S25. The first of these, the alcoholic precipitate (S23), showed some growth-promoting activity. However, its activity was less than that of the second, the ether precipitate (S22), in spite of the fact that the growth rate per week with each fraction apparently was the same. This follows from the fact that S23 was given in an amount equal to twice the liver equivalent of S22. The calcium salt from the ether filtrate (S25) did not differ significantly in activity from S23. It is of interest that the activity of S18 is approximately accounted for by the sum of the activties of S22, S23 and S25 when compared on the same liver equivalent basis.

The growth-promoting material of the ether precipitate (S22) apparently was destroyed by acid hydrolysis (S2H2). The weight increase of 7.0 gm. per week may be compared with that of 6.7 gm. found when S4 alone was administered. However, the purified fuller's earth filtrate (S13) after similar treatment was able to support growth at a rate of 12.7 gm. per week (S1H3). This may be compared with the rate of 19.5 gm. found with unhydrolyzed fuller's earth filtrate.

Mercuric acetate precipitation removes a very large amount of material which is nearly inert (S30). The greater part of the activity is found in the filtrate (S33). Moreover, the growth rate when both fractions are given is not significantly greater than that of the mercury filtrate alone.

The neutral ether extract (S19) was not only inactive of itself, but also failed to supplement in any way the activity of the ether precipitate (S19 + S22).

DISCUSSION

It is obvious that we are dealing with the same group of factors as Edgar, Macrae and co-workers. The successful adsorption of the activity on charcoal corresponds with the experience of Edgar and Macrae ('37) with the yeast filtrate

factor. The extraction of the activity from acid solution by amyl alcohol, and its return into dilute aqueous sodium hydroxide is analogous to the preparation of the liver filtrate factor by Edgar, El Sadr and Macrae ('38 b). The failure of mercuric acetate to precipitate any significant part of the liver filtrate activity (S33 + S30) is similar to the behavior of yeast filtrate factor ('37).

On the other hand, differences between the findings of the two laboratories have appeared. The growth rates reported by Edgar, Macrae and co-workers, with both yeast and liver supplements, are consistently higher than those which we find with the 95% alcoholic filtrate [2] and are about the same magnitude as those we find with cruder extracts. Moreover, there is a difference in the behavior of the liver extracts toward fuller's earth treatment. Together these facts suggest that the Liver Residue I of Edgar, El Sadr and Macrae ('38 b) may contain another accessory growth substance present in relatively low concentration in the 95% alcoholic filtrate.

The extraction of the activity from acid aqueous solution represents a new step in the concentration of the material necessary for rat growth. Continuous ether extraction recently was successfully applied by Elvehjem and collaborators to the problem of the chicken antidermatitis factor (Woolley, '38). However, Frost and Elvehjem ('39) have reported that such extracts gave irregular results when fed to rats and suggested that toxic products were generated in the process of acid ether extraction. The findings in this laboratory have been quite different, and we are unable to offer any explanation of this discrepancy. We are agreed that only a part of the activity is extracted by ether or the activity is extracted only very slowly, for after 48 hours' continuous extraction, a considerable activity remains in the aqueous phase (S27). Whether the activity of the two phases is due to the same or different substances is a problem which cannot be settled on the basis of present data. However, a difference in the

[2] A part, but not all, of this difference is explained by the fact that Edgar, El Sadr and Macrae have used only male animals whereas we have used both sexes.

properties of the active material in the ether extract from that of the original solution (S13) is indicated by the different stability to acid hydrolysis. These findings are especially significant in the light of reports from other laboratories (Schultz, '37, '38; Edgar, '37) which indicate a high degree of stability of the active substances of yeast extracts to acid hydrolysis. We are, therefore, led to the tentative hypothesis that the filtrate factor consists of at least one stable and one or more unstable substances.

A clue to the nature of the unstable material is obtained from the method of preparation of S22. The methods used in its preparation are similar to those employed by Snell, Strong and Peterson ('37) and by Williams and co-workers ('38) in the preparation of pantothenic acid, which markedly stimulates the growth of certain yeasts (Williams, '38), lactic acid bacteria (Snell, '38) and the diptheria bacillus (Mueller, '38). Through the kindness of Dr. Leo Rane, our fraction S22 has been tested for its growth-promoting activity on the diphtheria bacillus. This fraction could not contain a significant amount of free β-alanine, because the calcium salt of this amino acid is insoluble in alcohol. Moreover, this fraction was found to contain only a slight trace of amino nitrogen by the nitrous acid method. It was found that the preparation S22 could replace β-alanine in the synthetic medium, a property characteristic of pantothenic acid. This suggested that pantothenic acid might be the active substance of the S22 preparation.

In order to test this hypothesis, a sample of the calcium salt preparation was purified by the procedures of Williams ('38). These procedures involve alcohol precipitation, treatment with mercuric chloride in alcoholic and aqueous solution, and fractionation of an alcoholic solution of the purified calcium salt, by means of isopropyl ether precipitation. This purified material has been found to stimulate growth when administered in doses between 6 and 12 mg. weekly to each animal. It is not yet known whether or not smaller amounts may be equally effective. It appears likely, therefore, that

pantothenic acid is one of the substances necessary for rat growth.

There is not necessarily any discrepancy between this conclusion and the recent identification of pantothenic acid with the chick antidermatitis factor by Jukes ('39) and by Woolley, Waisman and Elvehjem ('39). Oleson, Bird, Elvehjem and Hart ('39) found that concentrates which were active in the prevention of chick dermatitis gave little improvement in the growth of rats. They found, however, that such concentrates brought about a rapid cure of the 'spectacled eye' condition.

Experience has shown that the growth-promoting ability of a given substance depends upon the basal dietary regimen—whether it is or is not the limiting factor. It is entirely possible, therefore, that a substance which simply cures a deficiency symptom in animals on one basal diet could actively stimulate growth in animals on a different basal diet. For these reasons, we believe that pantothenic acid is the active substance with which we are dealing in the calcium salt preparations.

The authors wish to express their gratitude to Dr. Guy W. Clarke of Lederle Laboratories, Inc., for his generosity in supplying the liver extracts used in these experiments, and to Dr. Merton C. Lockhart for assistance in large scale preparations.

SUMMARY

Albino rats grew only slowly when given a basal diet essentially free from B-vitamins, but supplemented by adequate amounts of pure thiamin and riboflavin and by fuller's earth adsorbate from liver extracts. The growth-promoting substances of the fuller's earth filtrate could be concentrated by extraction with amyl alcohol, adsorption and elution from charcoal and by continuous ether extraction of the acid aqueous solution. The activity of the ether extract could be concentrated further by fractionation of the brucine and calcium salts.

It is suggested that pantothenic acid is responsible for part of the growth-promoting activity of liver extracts.

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THE EFFECT OF PHOSPHORUS ON THE BIOLOGICAL ESTIMATION OF VITAMIN D ACTIVITY[1]

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TWO FIGURES

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In a previous publication (Morgareidge and O'Brien, '38), we have shown that the addition of vitamin D concentrates to skimmed milk results in an apparent total antirachitic activity for the mixture which is two to three times greater than can be accounted for on the basis of the concentrate added. In the light of experiments reported herewith, it becomes evident that milk phosphorus is the component which plays the dominant role in producing this enhancement effect. Furthermore, the data serve to reemphasize the fact that vitamin D content and total antirachitic potency cannot be used synonymously in any but the most strictly limited cases where supplementary factors are known to be absent. This necessary limitation is inherent in the standard curative assay procedure employing rachitic rats as the test animals.

EXPERIMENTAL

In order to maintain experimental conditions comparable to those employed for the assay of milk samples, it was desirable to feed the vitamin together with a source of phosphorus dispersed in an aqueous protein-containing medium. Five per cent solution of gelatin was found to satisfy the requirements since it provided a medium of very low phosphorus content

[1] Read before the thirty-third annual meeting of the American Society of Biological Chemists at Toronto, April 27, 1939.

(0.0015 mg. per cubic centimeter) in which vitamin D could be stably dispersed by adding a prophylene glycol solution of crystalline D_2. We have found that prophylene glycol solutions of vitamin D cannot be diluted with water alone but that the presence of some protective colloid (such as gelatin or milk protein) is necessary in order to produce stable dispersions of suitable potency for assay feeding.

The essential features of the assay technic may be briefly described as follows: Young rats from our standardized D-assay colony are weaned directly to the 2965 diet of Steenbock and Black ('25). A 21-day depletion period is allowed for the development of rickets. Prior to test-feeding, each animal is examined by radiograph of the left knee-joint and any failing to show uniformly satisfactory rickets are discarded. At the beginning of the test the rats average 40 days of age and 55 to 60 gm. in weight. The test-feeding is carried on for 8 consecutive days and final examination for degree of healing is made on the tenth day by the radiographic technic (O'Brien and Morgareidge, '38). Our standard test-group consists of seven animals and for every series of comparisons, four or five such groups are given graded levels of International Standard vitamin D solution diluted in corn oil and administered from calibrated dropping pipettes. The levels of standard fed are chosen to cover the range of 0.35 to 0.80 I.U. per day in steps corresponding to the ratio of $\sqrt{2}:1$. Differences in vitamin intake of this magnitude are easily distinguished and the response of our colony is such that this range of levels covers the most critical region of the healing curve. The material to be tested is fed simultaneously to four or five groups of rats in such amounts that the resulting healing matches that produced by the standard at at least two levels. If the approximate potency of the material is not known, a preliminary assay is run to establish the proper feeding levels.

In all the experiments reported here, the source of vitamin D was a single bottle of Drisdol (Winthrop Chemical Co.) which is crystalline D_2 dissolved in propylene glycol. Five

per cent solutions of BACTO gelatin (Difco Laboratories) were fortified with this material so that the necessary daily dose was contained in 4.0 cc. of gelatin solution. Using a total of ninety-eight rats in six independent assays, the potency of this sample was determined to be 6.7 I.U. per mg.[2] (average deviation, ±1.4) when fed dispersed with gelatin. The average number of units of healing observed plotted against the number of units fed per day are shown by the solid circles in figure 1. The dotted line is theoretical for a one-to-one correspondence.

In our previous work with vitamin D milks, the dilutions were always so arranged that the daily volume of milk fed was 4.0 cc. The average total phosphorus content of these samples was determined according to the method of Holtz ('29) through the cooperation of Mr. David Alling and found to be 1.0 mg. per cubic centimeter (±0.3 mg.). Therefore, we prepared 5% gelatin solutions containing sodium glycerophosphate calculated to contain 4.0 mg. of phosphorus in 4.0 cc. (based on actual analysis of the glycerophosphate). These phosphate-gelatin solutions were then fortified with varying amounts of vitamin D and the apparent potency of the Drisdol used was calculated from a series of three independent assays employing a total of 105 rats (exclusive of the standard animals). The value obtained was 22.7 I.U. per milligram (average deviation, ±3.4). When the actual amounts of vitamin D fed are compared with the observed healing levels in International Units, the open circles in figure 1 are obtained. Control experiments were run in which the gelatin solution alone and gelatin plus 8.0 mg. of phosphorus per day gave no healing. Thus, when 4.0 mg. of phosphorus were fed daily together with vitamin D, the apparent potency of the vitamin preparation was increased from 6.7 to 22.7 I.U. per milligram, giving an enhancement factor of 3.4 (which is numerically equivalent to the slope of the line

[2] Due to the fact that propylene glycol is very hygroscopic, the absolute potency will decrease with use if the sample is not carefully protected from atmospheric moisture.

drawn through the points obtained at the various healing levels).

Similar experiments were done to determine the effects of 2 mg. and of 1 mg. of phosphorus per day (one experiment each) and the results are shown by the barred and the half-solid circles, respectively, in figure 1. The slopes of these

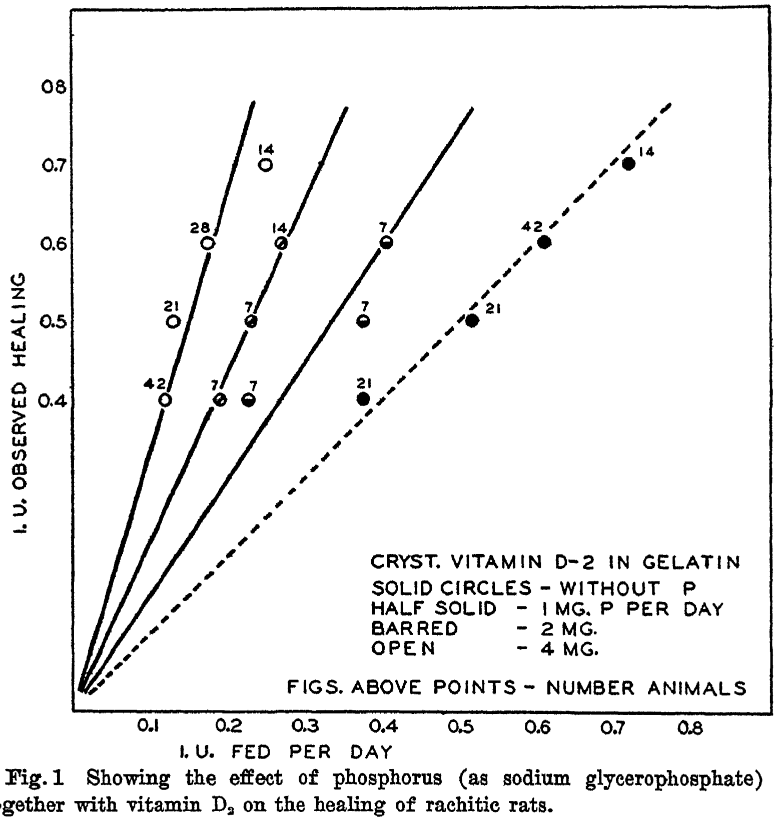

Fig. 1 Showing the effect of phosphorus (as sodium glycerophosphate) fed together with vitamin D_2 on the healing of rachitic rats.

lines can be designated as the 'enhancement factors' for their respective amounts of phosphorus (as sodium glycerophosphate) on the activity of vitamin D. When plotted as shown in figure 2, they are seen to fall on a straight line which is expressed by the equation:

$$E.F. = 1.0 + 0.58\,P \quad (1)$$

Since the total antirachitic activity of a mixture also depends on the amount of vitamin D present:

$$A = (1.0 + 0.58\,P)\,D \tag{2}$$

where A is the observed vitamin D activity, P the number of milligrams of phosphorus, and D the number of units of vitamin D.

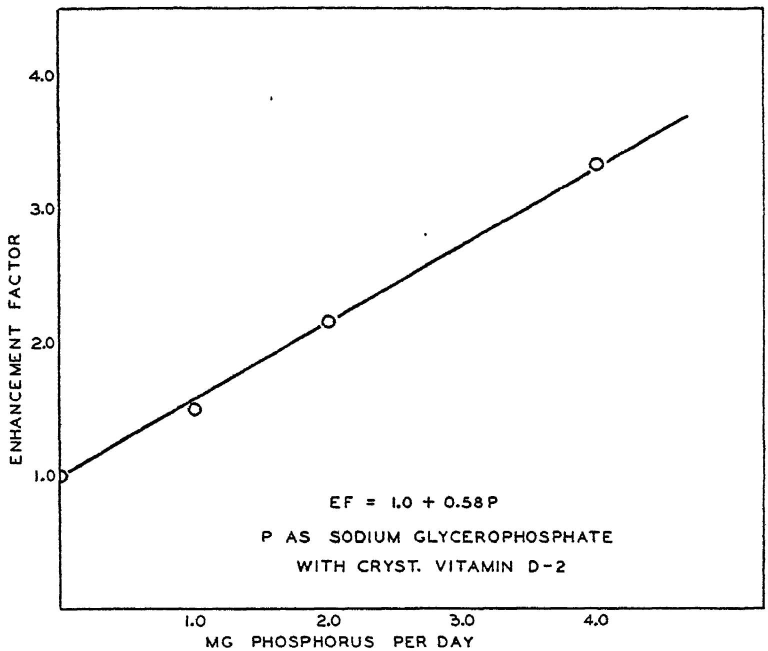

Fig. 2 Showing the relationship between the amount of phosphorus fed and the enhancement of vitamin D activity. In this chart, the points represent the slopes of the lines in figure 1.

We have also conducted some experiments to show that all types of phosphorus containing compounds do not yield the same enhancement factor. Both soluble and insoluble inorganic phosphates have been tested. Primary sodium phosphate (NaH_2PO_4) fed at a level equivalent to 4.0 mg. of phosphorus per day gives a factor of 3.5 (not significantly different from glycerophosphate). The insoluble phosphate tested was hydroxylapatite which was prepared by dissolving

calcium chloride in 5% gelatin solution and then adding phophoric acid to give a caluculated concentration of phosphorus of 1.0 mg. per cubic centimeter. The pH was maintained between 6.0 and 7.0 (Beckman glass electrode) during the addition of the H_3PO_4 by the simultaneous addition of sodium hydroxide. The finely divided amorphous precipitate of $Ca_{10}(PO_4)_6(OH)_2$ settled only slowly in the gelatin solution. A homogeneous mixture for feeding was obtained by stirring until the gelatin was near its setting point and then chilling rapidly. This form of phosphorus resulted in an enhancement factor of 2.2 at the 4.0 mg. per day level.

Values for the enhancement factor of the total phosphorus of milk have also been obtained under these same experimental conditions. These values have fallen in the range given by hydroxylapatite and are consistent with our earlier findings. However, the phosphorus of milk occurs in a variety of forms, both as soluble and insoluble organic and inorganic combinations. A complete analysis of the enhancement factor of milk has not yet been completed.

DISCUSSION

From a practical point of view, one of the most significant results of these experiments is the actual numerical relationship between the minimum effective level of vitamin D with and without added phosphorus. When fed the International Standard vitamin D solution alone, rachitic rats from our colony rarely show any traces of healing at levels below 0.35 I.U. per day for 8 days. This minimum level varies from colony to colony and from time to time in the same colony. For assay purposes we usually find 0.40 to 0.45 I.U. per day to represent the lowest practical dose. The fact that an intake of 4.0 mg. of phosphorus per day in addition to that obtained from the basal diet lowers this minimum effective level of vitamin D intake to the neighborhood of 0.1 I.U. is of importance. This is especially true when one considers that, to initiate healing, the rat requires twice this amount of phosphorus and nearly four times this much vitamin D when either is fed alone.

The problem of the bio-assay of vitamin D milks is of great interest to many. Obviously, it becomes necessary, in defining the antirachitic potency of milk, to recognize that it is determined by the product of the phosphorus by the vitamin D content. It is interesting to note in this connection that human milk contains only about one-fifth to one-sixth as much phosphorus as cow's milk. It seems reasonable that this fact may largely account for the very low apparent vitamin D content reported for breast milk. Outhouse, Macy and Brekke ('28) compared the antirachitic potency of human milk with that of cow's milk. They fed up to 40 cc. of human milk (the volume was reduced by evaporation) per day and found that it produced no appreciable increase in bone ash over rachitic controls. Thirty cubic centimeters of cow's milk, on the other hand increased the bone ash values from 31 to 41% (a net increase of 10%). The rachitogenic ration used was that of Osborne, Mendel and Park and the Ca/P ratios of the dietary intakes during both the rachitogenic and healing periods were carefully equalized. However, it may be calculated from their data that the phosphorus intake of the rats on human milk was nearly 8.0 mg. per day (Ca/P = 4–5:1). At the same Ca/P ratio, the rats on cow's milk were receiving 19 mg. of phosphorus per day. Shohl ('36), however, has clearly demonstrated that the absolute amounts of calcium and phosphorus in the diet are fully as important in characterizing its rachitogenic properties as are the ratios of these two elements. His data indicate that Outhouse and her associates used a diet in assaying human milk which was considerably more rachitogenic that that used for cow's milk.

Finally, it is necessary to point out that the rachitic rat is in a state of acute phosphorus starvation on the high-calcium, low-phosphorus diets commonly used in assay work. That this dietary ratio of calcium to phosphorus is of foremost importance in the production of rickets has long been recognized. It is not surprising, therefore, to find that even a partial restoration toward normal of the intake of these two elements has a profound influence on the efficiency with which vitamin D promotes the healing processes. At the

present time there is no experimental basis for assuming that the findings reported here with regard to vitamin D and phosphorus apply to any species other than the rat or to any other set of experimental conditions than those described.

SUMMARY

It has been found under conditions commonly employed for the biological estimations of vitamin D that phosphorus added to the vitamin supplement greatly enhances its apparent potency. The equivalent healing is found to be proportional to the product of the vitamin by the phosphrous fed. For example, 4.0 mg. of phosphorus per day for 8 days (as sodium glycerophosphate) enhances the potency of crystalline vitamin D_2 by a factor of 3.4 times. All phosphorus containing compounds do not possess the same enhancement factor. The significance of this relationship in the estimation of the antirachitic potency of phosphorus containing foods (such as milk) is pointed out.

ACKNOWLEDGMENT

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SOYBEAN HAY, ALFALFA HAY AND YELLOW CORN AS VITAMIN A SUPPLEMENTS IN COTTONSEED MEAL RATIONS FOR GROWING CATTLE [1]

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In a prior publication from this station (Halverson and Sherwood, '30) it was shown that the so-called cottonseed meal injury to cattle is due primarily to a lack of vitamin A in rations containing comparatively large amounts of cottonseed meal but without a leafy roughage. The investigation described here was begun in 1928 in order to confirm the earlier conclusion and to determine the amount of yellow corn, alfalfa hay or soybean hay required to prevent deficiency symptoms and to promote normal growth in young cattle, and also to determine the minimum daily carotene requirement for growth.

The syndrome of vitamin A avitaminosis is now well known (Jones et al., '26; Bechdel et al., '28; Kuhlman et al., '30–'32; Hart and Guilbert, '33). Several investigators have produced these symptoms in cattle by feeding cottonseed meal supplemented with feeds which contain little or no vitamin A. They have also been able to prevent or cure the condition by the use of good quality hay or other vitamin A carriers (Reed et al., '28; Bechdel, '31; Bechdel and Williams, '32; Copeland and Fraps, '32; Hart and Guilbert, '33; Kuhlman et al., '34–'36, and Bechdel and Skaggs, '35).

[1] Published with the approval of the acting director of the North Carolina Agricultural Experiment Station as no. 105 of the journal series.

Fraps and Treichler ('33), Russell et al. ('34) and Wiseman et al. ('38) have found that the vitamin A activity (carotene) in alfalfa hay is quite variable depending upon the grade or quality of the hay, the method of curing and the time in storage. Wiseman et al. ('38) state that the carotene content of U. S. no. 1 alfalfa hay varies between 19 and 121 mg. per kilogram. The no. 2 grade contains 12 to 20 and the no. 3 grade carries 1 to 11 mg. per kilogram.

Hilton et al. ('35) reported that field-cured young alfalfa hay had a vitamin A activity equivalent to 18 Sherman-Munsell units per gram and that their late alfalfa hay contained 20 units. They also found that young and late soybean hays respectively contained the equivalent of 36 and 8 Sherman-Munsell vitamin A units per gram.

Fraps ('31) found twenty samples of yellow corn to contain 2.5 to 8 Sherman-Munsell units vitamin A activity per gram. Wiseman et al. ('38) say that yellow corn contains 3 to 9 mg. carotene per kilogram, but they say that these figures include other pigments which go into the carotene fraction.

Guilbert and Hart ('35) found that the daily minimum carotene requirement of the bovine is 26 to 33 μg. per kilogram live weight when the carotene is supplied in alfalfa hay. A carotene intake at the level of 29 μg. per kilogram daily prevented or cured clinical symptoms and promoted normal weight increase, yet it resulted in no storage. When the intake fell below this level, night blindness reappeared and gains decreased. They also advanced the hypothesis that the vitamin A requirement is related to body weight rather than to energy requirement.

Ward et al. ('38) found that when alfalfa hay or carotene in cottonseed oil was used as the source of vitamin A, 12 to 14 μg. of carotene per pound of body weight per day was sufficient to prevent the symptoms of vitamin A deficiency in growing calves. Increased growth or improved well being in Holstein calves did not result when the carotene was supplied above 20 to 30 μg. per day per pound body weight. Work

with older animals indicates that heifers 1 to 2 years of age will probably show little or no carotene deficiency in the winter months if they have been on good pasture during the previous summer.

The work of Meigs and Converse ('38) indicates that the carotene requirement for successful reproduction is considerably higher than the minimum for growth or the prevention of night blindness.

EXPERIMENTAL

A series of five consecutive feeding trials or experiments was conducted. In the first experiment grade Shorthorn steers were used. High grade Shorthorn heifers were used in experiments II, III and IV. In the last experiment there were four Hereford steers, nos. 2, 3, 6 and 7; one Angus steer no. 5; two heifers, nos. 1 and 4 which were Hereford and Angus crosses and one Hereford and Shorthorn cross bred heifer, no. 8. The animals in all experiments were taken off of grass pasture in the late autumn and all except those in experiment V were given a ration of shelled white corn and soybean hay at a little above the maintenance level until placed on the experimental rations. The animals in all experiments except no. 1 were spring calves 7 to 9 months old. In experiment I they were approximately 20 months old when taken from pasture. They were put in the experiment 88 days later. The lengths of the preliminary feeding period in the subsequent experiments were 7, 33, 36 and 0 days respectively.

All of the animals were individually stall fed, but during the day they were allowed to run together in a paddock from which all grass, weeds and other feeds were carefully excluded. Daily records were made of the feed consumed and the animals were weighed fortnightly.

For better control in feeding, the concentrates and roughages were not fed separately but were ground and thoroughly mixed, enough of the ration to last approximately 3 weeks

being prepared at one time. The composition of the rations used is shown in table 1.

The alfalfa meal had a bright green color and was of excellent quality. While the alfalfa hay graded no. 3 it varied somewhat in quality for each experiment. This hay was locally grown and was mixed with fox tail (Chaetochloa glauca) and crab grass (Syntherisma sanguinaly). The soybean hay was of good quality and usually had a bright green

TABLE 1

Percentage composition of rations used

Ration no.	1	2[1]	3	4	5	6	7	8	9
Cottonseed meal (36% protein)	48	49.0	24.5	44.7	26	26	26	26	8.2
Cottonseed hulls	25	24.5	24.5	22.3	22	27	27	17	—
Beet pulp	25	24.5	24.5	22.3	25	—	—	—	—
Yellow corn (no. 2 or 3)	—	—	24.5	—	26	—	—	—	—
White corn	—	—	—	—	—	26	26	26	59.4
Alfalfa meal	—	—	—	8.9	—	—	—	—	—
Alfalfa hay	—	—	—	—	—	20	—	—	—
Soybean hay	—	—	—	—	—	—	20	30	32.4
Mineral mixture[2]	2	2.0	2.0	1.8	1	1	1	1	—

[1] Fed with 0.99 and 0.97 ounce cod liver oil daily 168 days to animals nos. 11 and 12 respectively; increased to 1.99 ounces daily for the next 226 and 227 days.

[2] Equal parts of calcitic limestone and steamed bone meal, common salt being always available.

color. Both kinds of hay were baled and stored in a rather dark barn loft.

In the last three experiments both the hay and the corn were sampled when first received. Later other samples of the hays were taken when they were being ground. These samples were kept in duplicate well filled sealed glass jars, in a refrigerator and dairy cooler until assayed for vitamin A by the rat growth method. International standard carotene was used as the reference standard in the assay. The results are given in table 2.

In computing the vitamin A intake of the animals in experiments I and II the following average values, in international units per gram, were used: yellow corn 2.5, alfalfa meal 11, alfalfa hay 7, soybean hay 19 and cod liver oil 600. The cod liver oil used was a grade sold for animal feeding and guaranteed to contain not less than 600 international vitamin A units per gram.

The earlier symptoms regarded as definite indications of avitaminosis A were a pronounced loss of appetite, a rough unkempt hair coat and listlessness. The eyes also were often affected at this stage. They became rather dry with a dull glassy appearance, followed by lacrymation and nyctalopia.

TABLE 2

Vitamin A activity of white corn, yellow corn, alfalfa hay and soybean hay used in experiments III, IV and V in international units of vitamin A per gram

Experiment	III	IV		V				
Month of exp. when sample taken	Initial	Initial	4th	Initial	3rd	5th	11th	14th
White corn	—	<0.5	—	0	—	—	—	—
Yellow corn	2.5	1.5	—	2.5	—	—	—	—
Alfalfa hay	10.0	3.0	5	11.0	—	6	5	7[1]
Soybean hay	22	10.0	20	17.0	14	22	22	19

[1] Composite of samples taken thirteenth to fifteenth months.

RESULTS

The results of the experiments are presented according to similarities in the vitamin A intake and the behavior of the animals.

Table 3 lists the data pertinent to the animals receiving the basal cottonseed meal ration no. 1, the rations containing yellow corn, nos. 3 and 5, ration 4 containing 8.9% alfalfa meal and ration 6 with 20% alfalfa hay.

It is probable that a part of the vitamin A reserves of the rather large steers in experiment I was used in the 88-day preliminary period in which they received a limited amount of shelled white corn and soybean hay. As a consequence they developed incipient symptoms of vitamin A avitaminosis

TABLE 3

Rations with a low vitamin A content

Ration	*Vitamin A supplement*	*Experiment*	*Animal*	*Vitamin A intake per kilo body weight per day*		*Body weight*			*Average daily gain*		*Length of period*		*Deficiency symptoms at end*
				Fore-period[1]	Entire period	Initial	End of fore-period	Final	Fore-period	Entire period	Fore-period	Entire period	
No.		No.	No.	I.U.	I.U.	lbs.	lbs.	lbs.	lbs.	lbs.	days	days	
1	None	I	1	0	0	835	1205	1025	2.64	1.01	140	187	Severe
1	None	I	2	0	0	823	1005	865	1.62	0.20	112	210	Severe
1	None	I	3	0	0	825	975	650	1.34	—	162	167	Died
1	None	I	4	0	0	745	930	720	1.65	—	112	165	Died
1	None	I	5	0	0	835	1035	940	1.79	0.56	112	186	Died
1	None	II	7	0	0	395	715	655	1.63	1.03	196	252	Severe
1	None	II	8	0	0	275	530	505	1.52	1.10	168	210	Severe
3	24.5% yellow corn	II	9	16	15	370	685	690	1.89	1.03	168	196	Severe
3	24.5% yellow corn	II	10	16	15	280	550	522	1.29	1.07	210	226	Severe
5	26.0% yellow corn	IV	1	11	9	327	625	575	1.93	1.11	154	224	Severe
5	26.0% yellow corn	IV	2	12	11	318	640	610	2.09	1.68	154	174	Severe
5	26.0% yellow corn	V	1	—	11	475	—	630	—	0.69	—	234	Moderate
5	26.0% yellow corn	V	2	15	14	310	475	500	1.27	1.07	130	178	Severe
4	8.9% alfalfa meal	II	13	—	23	320	—	1000	—	1.73	—	394	None
4	8.9% alfalfa meal	II	14	23	22	315	915	897	1.43	1.06	420	453	Severe
6	20.0% alfalfa hay	IV	3	21	19	378	980	985	1.78	1.64	338	371	Incipient
6	20.0% alfalfa hay	IV	4	25	28	245	675	680	1.71	1.35	252	323	Severe
6	20.0% alfalfa hay	V	3	—	28	465	—	975	—	1.19	—	430	None
6	20.0% alfalfa hay	V	4	—	33	325	—	535	—	0.66	—	318	Moderate

[1] The fore-period is that part of the experimental period in which the animal was apparently normal before symptoms of vitamin A deficiency became manifest.

in a shorter time than the smaller heifers in experiment II, which were put on the experimental basal ration 1 week after removal from grass pasture.

The time required for symptoms to develop in the six head fed approximately 25% yellow corn in their ration did not differ significantly from that of the comparable negative controls (nos. 7 and 8, experiment II) even though they got 9 to 15 international units of vitamin A per kilogram body weight daily. When 25% of yellow corn is the sole source of vitamin A in a cottonseed meal ration it does not supply enough of this essential to protect young cattle or even to postpone materially the time of nutritive failure.

Four of the six head fed 8.9% alfalfa meal or 20% no. 3 alfalfa hay exhibited symptoms of vitamin A deficiency in 252 to 420 days and two survived 394 and 430 days without failure. Since the vitamin A intake of these animals was 19 to 33 units per kilogram per day, alfalfa hay or meal supplying these amounts of vitamin A delays the time of failure but does not offer safe protection after 8 months.

None of the twelve head in table 4 fed the cottonseed meal ration supplemented with approximately 1.5 ounces of cod liver oil per day or with 20% or more of soybean hay exhibited any symptoms of vitamin A deficiency in over a year of the experimental regime. The least amount of vitamin A ingested by any of these animals averaged 59 international units daily per kilogram of body weight. At this level of vitamin A intake the average daily gains were somewhat greater than those listed in table 3 for the entire period although the animals receiving sub-minimal amounts of vitamin A were able to make satisfactory gains during the earlier part of the experiment before their reserves had been exhausted.

The results shown in tables 3 and 4 are in excellent agreement with those of Guilbert and Hart ('35) and of Ward et al. ('38). The former found the minimum daily carotene (in alfalfa hay) requirement to be equivalent to 43 to 55 international vitamin A units per kilogram body weight and the

TABLE 4

Rations with a high vitamin A content

Ration no.	2		7				8				9	
Vitamin A supplement	Cod liver oil		20% soybean hay				30% soybean hay				32.4% soybean hay	
Experiment no.	II		IV		V		IV		V		II	
Animal no.	11	12	5	6	5	6	7	8	7	8	8a[1]	15
Vitamin A intake, I.U. per kilogram live weight per day	81	90	59	60	82	89	87	85	110	138	162	156
Initial weight, lbs.	350	280	328	308	450	345	352	275	445	405	207	310
Final weight, lbs.	950	960	1055	940	1052	980	1035	868	1038	740	595	1000
Gain, lbs.	600	680	727	632	602	635	683	593	593	335	388	690
Average daily gain, lbs.	1.52	1.56	1.95	1.67	1.40	1.45	1.83	1.57	1.39	0.64[2]	1.33	1.58
Days fed	394	435	373	378	432	439	373	378	437	521	297	436

[1] 8a was a calf of no. 8 experiment V. The record presented here starts at the time the calf reached the age of 171 days although since birth it had not received any feed except its mother's milk and ration no. 8.

[2] This heifer was stunted because of a gestation and lactation period.

values of the latter are equivalent to 44 to 52 units per kilogram for carotene in oil.

The beef animals receiving 20% soybean hay in their ration reached a practical slaughter weight with a satisfactory degree of finish in 12 to 14 months without any evidence of vitamin A deficiency. So far as rate of gain and physical well being were concerned 30% soybean hay did not yield results that were superior to those obtained when 20% was fed although the estimated daily vitamin A intake per kilogram body weight was considerably below the five to ten times the minimal for the prevention of night blindness which Guilbert et al. ('37) recommended as a desirable minimum for practical purposes. The results from the one animal on ration 9 containing 32.4% soybean hay and from the two whose basal ration was supplemented with cod liver oil were very similar to those from the animals given 20 or 30% soybean hay.

These observations indicate that the minimum for practical purposes proposed by Guilbert and co-workers may be too high if only the immediate needs of the animal are considered. On the other hand, it is quite possible that a larger vitamin A intake is necessary, especially for lactating cows, to insure maximum storage in the body and a high vitamin A activity in the milk.

Heifer 8, experiment V, was pregnant when placed on the experiment, although only about 10 months of age. On the two hundred and forty-ninth day of the experiment she gave birth to a normal calf which she nursed successfully while continuing on the cottonseed meal ration containing 30% soybean hay. During the gestation period her daily vitamin A intake averaged 109 international units per kilogram of body weight, but because of a proportionately greater appetite in the lactation period she averaged 138 units per kilogram per day for the entire feeding trial. Neither the dam nor her calf, which was continued on the experimental ration, showed any signs of vitamin A deficiency.

The details for the eight animals in experiment III are not given because the experiment was terminated by fire at 140

days. In this trial, which was essentially the same as experiments IV and V, no evidence of vitamin A deficiency was manifest although two head received only 18 units of vitamin A per kilogram per day with a daily gain of 2.09 pounds compared to 1.92 for the other six head. This result is further evidence that weaned calves may be safely carried at least 4 months on rations containing large amounts of cottonseed meal and inadequate amounts of vitamin A when they have previously been on summer pasture.

Of the nineteen animals listed in table 3 three died, two did not develop deficiency symptoms and were slaughtered and four received therapy treatments the results of which are not pertinent to the present discussion. The rations of the remaining ten head were modified by the addition of more vitamin A by feeding cod liver oil to four head, alfalfa hay to two and soybean hay to four head. Increasing the alfalfa hay in ration 6 from 20 to 30% or the substitution of 10 or 20% soy bean hay for an equal amount of cottonseed hulls in ration 5 converted these inadequate diets into rations which cured the symptoms of vitamin A deficiency and enabled the cattle to make satisfactory gains in weight.

CONCLUSIONS

A ration for cattle composed largely of cottonseed meal and cottonseed hulls is deficient in vitamin A precursors. Because of its low vitamin A activity there is no practical level at which yellow corn may be incorporated in a cottonseed meal-cottonseed hulls ration as an adequate vitamin A supplement. No. 3 alfalfa hay is a rather poor source of carotene. When incorporated in the basal ration at a level of 20% of the entire ration it does not supply the minimum vitamin A needs of young growing cattle. Probably 50 to 60% of no. 3 alfalfa hay in the ration will furnish more than the minimum requirement for vitamin A precursors.

The soybean hay used in these experiments had approximately twice the vitamin A activity of the alfalfa hay.

Rations containing 20% soybean hay were adequate for growth and the physical well being of young beef cattle. When 30% was present in the ration one heifer had a successful gestation and lactation period.

These results are in accord with the findings of Guilbert and Hart ('35) and of Ward et al. ('38) that the minimum daily carotene requirement of cattle is equivalent to approximately 43 to 55 international vitamin A units per kilogram of body weight.

In the cotton belt where these feeds are comparatively cheap a ration composed of cottonseed meal, cottonseed hulls and a good hay in sufficient quantity is a practical one for growing cattle.

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THE FOOD OF THE PRESENT-DAY NAVAJO INDIANS OF NEW MEXICO AND ARIZONA

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Between 1932 and 1935 a study was made of the food habits of the Maya Indians of Yucatan (Benedict and Steggerda, '36) in which it was found that their food was chiefly carbohydrate (maize). This was challenging, as the basal heat production of the Maya averages 5 to 8% higher than the commonly accepted standards for white men living in the northern part of the United States (Williams and Benedict, '28; Shattuck and Benedict, '31; Steggerda and Benedict, '32). For comparative purposes it was desired to have information regarding the diet and the basal heat production of other Indian groups. Consequently between 1934 and 1936 a study was made of the food habits of the Navajo Indians, chiefly in the southern part of the Navajo reservation in New Mexico and Arizona, but up to the present time a study of the basal heat production of the Navajo has not been feasible. Sixty-six samples of foods in the Navajo's daily diet, both uncooked and cooked, were collected for analysis as to their fat, nitrogen and water content, and their energy value. In addition, data were secured with regard to the character and the amount of food eaten per Navajo family per week.

The Indians who supplied us with food samples and information regarding their food habits were living in hogans on the reservation. They were relatively pure Navajos, although it would be impossible to say with assurance that

any Indian is pure Navajo or to describe the genetic constitution of this group. A Navajo interpreter relayed our questions and in turn secured the information for us.

COMPOSITION AND ENERGY VALUE OF NAVAJO FOODS

The food samples were placed in pint, 'quick seal' metal cans with press-in covers. These were sealed air-tight and shipped to the Nutrition Laboratory in Boston for analysis. No preservative was added to those samples that were dry when collected. Decomposition of the samples that were stored while fresh was guarded against by sprinkling a few grams of benzoic acid over the top of the sample and mixing it with the contents of the can, the food and the acid occasionally being ground in a food chopper to obtain more thorough mixing. In some instances a few drops of formaldehyde were added instead of the benzoic acid. In Boston each sample was ground and placed in an oven at 60° C., after which it was exposed to room air for 24 hours to take on moisture from the atmosphere and was then weighed. This process was carried out a second time, and unless the two air-dry weights thus obtained for each sample (weighed to the nearest 0.01 gm.) agreed within 1%, the drying process was repeated.

The oxy-calorimeter (Benedict and Fox, '25; Benedict, '29) was used to determine the heats of combustion of the air-dry samples. It was assumed that each liter of oxygen consumed during the combustion of the food sample in the oxy-calorimeter represented 4.825 calories. Correction was made for the energy value of the benzoic acid (6.3 calories per gram—Benedict and Fox, '25) in those instances where it was used as a preservative, but no correction was made for the slight loss (10%) of benzoic acid in the air-drying process. The fat content was determined by ether extraction. When benzoic acid had been added to the sample, it was assumed that it was extracted by the ether during the analysis and that none of it escaped in vapor form. The weight of the benzoic acid was, therefore, deducted from the weight of the apparent

total fat in the sample. The nitrogen content was determined by the usual Kjeldahl method. No corrections were made for the formaldehyde used as a preservative. In those instances where sufficient sample was collected, the analyses were made in duplicate, and if the results did not agree within 2% of the total value found for oxygen and 5% of the total values found for nitrogen and fat, further analyses were made until agreement within these limits was obtained.

The results are reported in table 1 on the basis of air-dry material, but the percentage losses in weight of the foods brought from the condition as collected to an air-dry condition are also reported, so that the percentages of nitrogen and fat and the calories per gram can be calculated on the basis of the fresh weight, if desired. The absolute water content of the air-dry material (heated at 100°C.) is likewise reported in those instances where sufficient sample was collected to permit this determination. In some instances, although repeated determinations were made of the same factor, well-agreeing results were not obtained. In other instances only one determination or no determination of a particular factor could be made, owing to insufficient sample. These results are reported, although it is recognized that they have not been corroborated. For comparison with the yellow and the blue Indian corn, analyses were also made of Navajo, Maya, and Illinois white corn.

The fat and the nitrogen content of the different foods varied considerably, as would be expected, but the energy content was in most instances between 3.5 and 4.5 calories per gram of air-dry matter. The meats, which contained considerable fat, and also some of the native berries and seeds had higher energy values. Comparison with analyses of Maya foods (Benedict and Steggerda, '36) is possible in the case of the Maya white corn, the squash and the Chile pepper. This comparison indicates that, although the fat and the nitrogen content of these Maya and Navajo foods differ somewhat, the energy values of the foods are essentially the same.

TABLE 1

Analyses of typical Navajo foods

FOOD	WEIGHT LOST IN DRYING TO AIR-DRY CONDITION [1]	ON AIR-DRY BASIS [3]			
		Moisture [2]	Fat	Nitrogen	Calories per gram
	%	%	%	%	
Dry corn, roasted corn, corn flour					
Indian corn, yellow and blue	4.3	5.5	5.8	1.5	4.3
Navajo white corn	0.8	4.7	5.9	1.4	4.2
Maya white corn	3.0	5.0	4.1	2.0	4.3
Illinois white corn	4.8	3.8	4.3	1.4	4.2
Roasted corn	5.6	2.9	5.7	1.6	4.2
Roasted corn	7.4	2.9	6.0	1.7	4.4
Parched corn	3.9	4.1	5.2	1.8	4.2
Corn flour	0.0	7.9	5.6	1.7	4.1
Breads, dumplings, and mush					
Fried bread	18.7 [4]	9.6 [4]	5.2	2.2	4.1
Baked bread	24.6 [4]	7.7 [4]	1.9	1.9	3.8
Baked blue corn cakes	5.6	3.2	3.8	1.7	4.2
Wheat bread	4.2	4.8	5.1	2.4	4.2
Navajo yeast bread	43.0 [4]	6.7 [4,5]	0.0	4.4	4.1
White bread	6.8	2.5	3.1	2.4	4.4
Green corn tamale bread	...	..	...	1.9	3.9
Green corn tamale bread	4.1	3.7	4.2	1.7	4.2
Ash corn bread	3.0	4.4	4.1 [5]	1.7	4.1
Milk corn cakes	8.1	..	3.3 [6]	1.7 [6]	4.2 [6]
Poured bread	4.2	4.3	3.8	1.7	4.2
Sprouted wheat corn bread	3.7	3.4	5.2	1.8	4.3
Navajo corn cake (alkandt)	4.2	2.2	4.7	1.7	4.4
Green corn bread	4.1	4.3 [5]	3.2	1.8	4.1
Blue paper bread	3.9	4.5	2.8	1.6	4.1
Boiled blue corn cakes (dumplings)	6.8	3.0	3.3	1.7	4.3
Corn mush with cedar ash	63.7 [4]	6.7 [4]	0.05	1.8	4.1
Corn mush and salt	86.4	..	0.0 [6]	1.5	3.9
Vegetables					
Dried squash	0.0	..	1.0 [5]	0.4	3.5
Dried squash	...	..	2.5	0.4	3.6
Pinto beans	3.7	5.2	1.9	3.1	4.1
Wild potatoes and clay [7]	24.9	2.9	0.2	0.7	..
Onion	87.9	..	1.5	1.3	3.6
Wild carrot [8]	...	..	2.5	1.0	..
Chile pepper	0.0	..	...	2.1 [6]	5.3
Wild celery [8]	...	..	...	2.4 [6]	3.4 [6]
Fruits, nuts, and berries					
Dried Yucca	...	..	1.2	0.3	3.5
Dried Yucca	7.3	9.8	1.1	0.3	3.6
Dried peaches	...	6.5	1.4	0.9	3.6
Dried muskmelon	4.2	6.3	1.0	0.6	3.3

TABLE 1 (*continued*)

FOOD	WEIGHT LOST IN DRYING TO AIR-DRY CONDITION [1]	ON AIR-DRY BASIS [3]			
		Moisture [2]	Fat	Nitrogen	Calories per gram
	%	%	%	%	
Fruits, nuts, and berries (continued)					
Dried watermelon	6.3	21.4	1.5	1.4	3.4
Pinon nut butter	0.7 [4]	6.0 [4]	54.4	2.1	6.3
Pinon nut butter	2.8	3.2	24.0	1.7	5.5
Acorns [8]	...	6.7	5.8	1.7	4.1
Red Squaw berries	3.1	..	16.7 [6]	1.7	5.3
Wolf berries [8]	...	12.0	4.4	2.1	4.2
Choke cherry cakes	5.5	3.2 [5]	6.2	0.7	4.4
Ground cherries [8]	...	9.9 [6]	12.0 [6]	2.5 [6]	4.8 [6]
Silver nightshade berries [8]	...	..	...	2.4 [6]	5.0 [6]
Seeds of grasses and weeds					
Thistle seed	0.0	..	1.6	2.2	4.1
Dried seed	0.0	..	7.4 [5]	2.6 [6]	4.3
Weed seed	0.0	..	...	4.1 [6]	6.3 [6]
Sunflower seed [8]	...	..	...	3.2 [6]	5.9 [6]
Tumbleweed seed [8]	...	..	0.1 [6]	2.1 [6]	3.9 [6]
Sage seed [8]	...	..	0.3 [5]	2.8 [6]	4.2 [6]
Indian rice grass or Indian millet seed [8]	...	..	...	1.1 [6]	3.9 [6]
Tansy mustard [8]	...	..	2.2 [6]	3.9 [6]	5.6 [6]
Narrow leaf grass [8]	...	..	...	2.8 [6]	3.9 [6]
Rocky Mountain bee plant [8]	...	..	1.4 [5]	3.5	4.6 [6]
Rocky Mountain bee plant [8]	...	..	1.6 [6]	3.4	3.9 [6]
Meats					
Broiled mutton	49.9 [4]	4.8 [4]	10.8	11.6	5.4
Large intestine	1.6	2.3 [5]	82.1	2.5	8.9
Stomach of goat	1.6	5.8	55.8 [5]	4.4	7.2
Stomach of goat	2.7 [4]	3.5 [5]	38.7	7.7	6.6
Skin of sheep	36.8	..	12.4	10.5	5.5
Blood wurst	56.2 [4]	3.3 [4,5]	40.3	5.6	6.8
Miscellaneous					
Juniper ashes [8]	...	1.7	0.0 [6]	0.2 [6]	..
Clay [8]	...	9.2	0.7	0.0	..

[1] Loss in weight of food when brought from condition as collected to air-dry condition.

[2] Percentage of moisture found in air-dry food in bringing it to an absolutely water-free condition.

[3] See explanation on pages 298 and 299.

[4] Includes benzoic acid.

[5] Average of values not agreeing within 5%.

[6] One analysis only.

[7] This sample, when air dry, contained 76% ash.

[8] Samples collected by Miss Stella Young (see p. 304).

QUANTITATIVE STUDY OF THE FOOD HABITS OF THE NAVAJO

Since the coming of the Spaniards, mutton (from either the sheep or the goat) may be called the chief food of the Navajo. On some small sections of the reservation beef supplants mutton, but this is not generally true. In contrast to the habits of the white man, the Navajo consumes all parts of the sheep or goat, including most of the alimentary canal (the different parts of the stomach and the intestines). The male reproductive organs, the brain, the soft parts of the hoofs, the ligaments of the legs, occasionally the skin after the hair has been removed, the blood and the eyes are all used. The only parts not eaten are the contents of the digestive tract, the bladder, the gall sack, the bones, the horns, and the hair. Perhaps, because the Navajo does consume practically all parts of the animal, he may have reached a dietary balance, which perfects him for life in his surroundings and which gives him a nutritional reserve resulting in his excellent physique as well as his excellent teeth (Steggerda and Hill, '36).

Very little of the corn plant is wasted. In the early summer the half-formed ears are boiled in milk and are completely eaten, even the cob. Some wheat is raised by the Navajos where they have sufficient water and some is bought from the traders or bartered from the Zuni Indians. Juniper ashes are used in place of baking powder and salt, as a flavoring for mushes and other foods and as a medicine. Clay is mixed with such foods as the wild potato and the tomatillo to counteract the unpleasant astringent effect of these foods on the mouth. Fried bread is used considerably, although it is not the main food. Baked bread is eaten freely, especially in winter. An adult man will eat three cakes (240 gm.) at one meal. The seeds of several kinds of grasses were once used for food, but very few of these seeds are used now. In times of great scarcity, any seed that could serve as a food was doubtless so used.

The quantitative study of the Navajo's food habits was not so complete and well controlled as was that made in Yucatan

for the Maya. However, the following information was secured from five reliable Navajo families concerning the amount of food eaten by each family. A family of twelve (five adults and seven children) consumes one adult goat each week or about 23 kg. of meat. The choice pieces of meat last about 3 days. During the other 4 days of the week the miscellaneous parts, intestines, feet and head, are consumed. In addition, bread, vegetables and coffee are eaten. A family of eight (three adults and five children) consumes the choice pieces of a goat in 5 days and for about 3 days thereafter the miscellaneous parts. This family also uses 11 kg. of flour every 3 days, but when much other food is available, such as vegetables, the flour lasts about 1 week. This particular family does not eat many vegetables but uses 0.5 kg. of coffee in 3 days and 2.5 kg. of white sugar each week.

Another family of ten (three adult women, two adult men and five children) consumes one goat per week, one sack (11 kg.) of wheat flour every 3 days (rarely does it last 4 days), 0.9 kg. of coffee each week, 2.5 kg. of white sugar each week and one can of condensed milk when the goats are not milking. However, from April to September this family uses from 3 to 8 liters of goat's milk per day, either as milk, butter or cheese. Another family of ten (six adults and four children) consumes one goat in 3 days. When the meat is gone, another animal is killed. For this family, 11 kg. of wheat flour last about 2 weeks. The informant added that this family uses considerable corn flour also, but little sugar. A family of fifteen, mostly adults, consumes one large mutton (25 to 30 kg.) every 2 days. In addition, corn, potatoes, bread, dried peaches and prickly pears are eaten.

In general, the average Navajo family of six or seven people consumes one goat, 10 kg. of flour, 2.5 kg. of white sugar and about 1 kg. of coffee every week, in addition to such vegetables as they may have. If available, the Navajo would make meat, chiefly mutton, 60 to 80% of his diet. The Navajos rarely keep chickens for food or eat eggs. The Navajos consume rabbits, the cottontail and the jack rabbit, and prairie dogs.

Deer and antelope used to be hunted in the mountains for food, but there are no deer now in the Navajo reservation and the antelope are protected by law. To a considerable extent the Navajos are dropping their old food habits and adopting the habits of the white man. For example, they are now turning to white flour instead of corn flour and they do not raise so many potatoes, as they buy from the trading posts. Also they buy considerable bacon in which to fry potatoes.

SUMMARY

Sixty-six samples of the commonest foods in the daily diet of the Navajo Indian of New Mexico and Arizona were analyzed with reference to their moisture, fat, nitrogen and energy content. In spite of the variability in fat and nitrogen content of the different foods, the energy content was relatively constant in most instances (between 3.5 and 4.5 calories per gram of air-dry matter). Higher energy values were found for the meats and some of the berries and seeds, which had higher percentages of fat.

The diet of the Navajo Indian consists chiefly of mutton in contrast to that of the Maya Indian of Yucatan, which is predominantly corn. Practically all parts of the animal (goat and sheep) are eaten, including most of the alimentary canal.

A study was made of the character and amount of food eaten by five Navajo families per week. The average family (six or seven persons) eats one goat each week, besides about 10 kg. of flour, 2.5 kg. of white sugar, 1 kg. of coffee and such vegetables as may be available.

ACKNOWLEDGMENTS

The majority of the Navajo foods were collected with the assistance of the late Mr. Wallace Peshlakai. Sixteen samples of food materials were kindly given to us by Miss Stella Young, head of the Home Economics department, Wingate Vocational High School, Fort Wingate, New Mexico, from her many years' collection of the seeds and fruits of native plants used by the Navajos for food. Thanks are especially

extended to Mr. and Mrs. Alexander Black of Fort Defiance, Arizona, for their thorough and conscientious checking of field notes and for critical reading of our manuscript. The determinations of the moisture content of the food samples were made by R. C. Lee and M. Stankard, the analyses for fat and nitrogen content by M. Stankard and the analyses for energy content by B. James.

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THE HEAT PRODUCTION OF THE FASTING RAT IN RELATION TO THE ENVIRONMENTAL TEMPERATURE [1]

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TWO FIGURES

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A study of the literature dealing with the heat production of the albino rat as affected by environmental temperature indicates that the critical temperature has not been well established, that the range in temperature over which the metabolism of this animal is constant has not been determined and that the percentage of heat eliminated by evaporation of water has not been convincingly shown to vary consistently with the environmental temperature.

Work reported by Benedict and MacLeod ('29 b) shows a constant rate of decrease in heat production as the environmental temperature is raised from 10°C. to 28°C. The graph showing the average trend of the metabolism is, unfortunately, not extended beyond 28°C. although it was considered from data not shown that, "at 28°C. and above the metabolism was essentially constant . . . ." The authors must have felt somewhat uncertain about this, however, for they also say in the same paper, "It is not impossible that temperatures higher than 28°C. may prove to lower the basal metabolism still further." No attempt was made to establish the range of temperature throughout which the metabolism remained constant.

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Goto ('23) measured the total heat and water vapor produced by rats exposed to temperatures ranging from 5°C. to 33°C. He found the minimum heat production at 28°C. with a higher heat production prevailing above as well as below this temperature. His observations were made on four rats during the first and also during the second day of fast. Though one would expect the percentage of heat eliminated by the evaporation of water to increase steadily and consistently with rise in environmental temperature, his data, especially those of the second day of fast, show considerable lack of consistency. For instance, one rat which eliminated 20.7% of its heat by vaporization of water at an environmental temperature of 25°C. eliminated only 12.8% at 28°C. Wesson ('31) considers that the basal metabolism of rats should be determined at 28°C. Schwabe and associates ('38) consider the range of thermal neutrality to be 28°C. to 30°C. Males and Magaz ('29) found the metabolism of fasting rats to be constant from 30 to 33°C. and only slightly variable throughout a range in temperature from 29 to 33.5°C. Prior to the measurement of metabolism the rats were kept at a temperature of 8 to 10°C., feed being withdrawn 10 to 14 hours before the experiment began. Two preliminary periods of ½ hour each followed; during the first one the rats were kept at an environmental temperature of 13 to 15°C. and during the second, at the temperature of the experiment.

Terroine and Trautmann ('27) working with many species of animals found that the minimum heat production with rats occurred at an environmental temperature of 33°C. Only two rats were used to secure a very limited number of observations at a given temperature.

By the use of animals which were similar as to age, weight and sex, and in the postabsorptive state, the primary purpose of this study was to determine 1) the critical temperature for the albino rat, 2) the range in environmental temperature over which the metabolism remains constant, and 3) the percentage of heat eliminated by the evaporation of water. In order to accomplish these objectives, it was considered neces-

sary to accumulate enough reliable data thoroughly to establish each individual point of observation with a reasonably small probable error, in order that the significance of the differences in findings at different temperatures could be statistically evaluated. It was felt that the short periods of observation ordinarily used at many laboratories are quite unsatisfactory, on account of the possibility of technical errors as well as the unavoidable physiological lag of the animal in response to a change in environmental temperature.

The open circuit Haldane method of procedure was followed, the oxygen absorption during 8 hours being weighed as the sum of the weights of CO_2 and water vapor eliminated minus the loss in weight of the rat and container. The CO_2 and water were weighed at intervals of 2 hours, the measurement for the first period of 2 hours being excluded. Thus, the respiratory quotient and the total oxygen consumption were derived from 8-hour periods, while the hourly heat and water vapor were derived from the carbon dioxide and water vapor of the last 6 hours.

The water produced was absorbed in glass-stoppered U-tubes containing pumice saturated with H_2SO_4, and the CO_2 was absorbed by flake NaOH mixed with pumice in similar tubes as described by Forbes ('39). These tubes were weighed on the ordinary chemical balance.

The food was withdrawn 15 hours before the start of the experiment, or 17 hours before the beginning of the 6-hour period of measurement of heat production and water vapor.

The rat was put into a screw-cap bottle of about 4 liters capacity, and after weighing to the nearest milligram on a chainomatic balance, the bottle containing the rat was immersed in a water bath, the surface of the water coming to within about 1 inch of the top of the bottle. Two lead weights attached to the bottle while in a water bath caused it to rest securely on a false bottom of hardware cloth.

Two brass tubes soldered to the metallic bottle caps served as inlet and outlet for the air current. The inlet tube went nearly to the floor of the bottle while the outlet extended only

an inch below the cap. Dry CO_2-free air was supplied at the rate of 2 liters per minute, this rate of flow being measured by means of a previously calibrated air-speed indicator.

A short thermometer fastened to the longer brass tube was used to indicate the temperature of the air within the bottle. The heat from the animal raises the air in the bottle to a temperature somewhat above that of the surrounding water bath. For instance, when the bath temperature was 4.0°C. the bottle temperature was 7.5°C.; when the bath temperature was 24.0°C. the bottle thermometer read 25.0°C. The difference between the temperature of the bottle and bath was 0.5°C. at a bath temperature of 27.5°C., and became progressively smaller at higher temperatures. The thermometer was read after raising the bottle out of the bath (permitted by flexible rubber tubing connections), and while holding the bottle at such a height that the top of the mercury in the thermometer was level with the eye. If the animal was resting against the bulb of the thermometer or was very close to it, the reading was improperly high and was not recorded. Otherwise the bottle temperature was as constant as that of the bath. The temperature of the air in the bottle was considered to be the environmental temperature to which the rat was exposed.

The rat rested upon a floor of ½ inch mesh hardware cloth supported about 1½ inches above the bottom of the bottle. The bottom of the bottle was covered about ½ inch deep with paraffin oil. As shown by trial, the amounts of water and CO_2 coming from the excreta covered with the oil were entirely negligible. Water evaporated from the urine clinging to the screen floor would give results too high for the percentage of heat eliminated by the evaporation of water, but would cause no error in the determination of the total heat production. However, in this connection it was observed in the few cases when no urine or feces were voided, that the results were indistinguishable from those when urine was present. Though urine was voided in most of the 8-hour periods, at all tempera-

tures, feces were rarely found after the periods conducted at 28°C., 30°C. or 31°C.

The water bath, the chamber and the use of oil to prevent evaporation of water from the excreta were devised by E. B. Forbes, and the bath, with accessory equipment, was constructed by Precision Scientific Co.

The bath was connected with an insulated tank containing ice water during experiments conducted below room temperature and hot water during experiments conducted above room temperature (fig. 1). A thermostat and solenoid valve operated to take water from the reservoir of cold (or hot) water

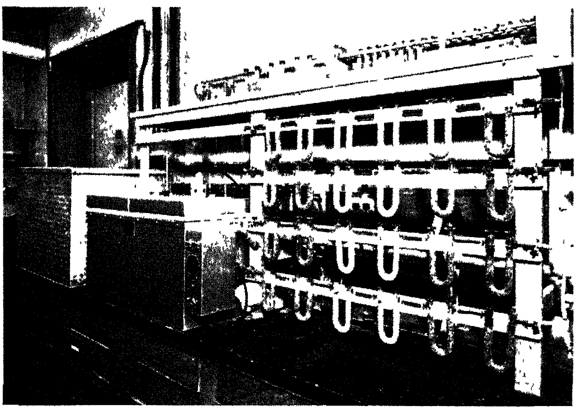

Fig. 1 Constant temperature bath and accessory equipment.

when needed to maintain the temperature of the bath constant to within ±0.4°C. or less. A pump circulated the water within the bath for the equalization of its temperature. The bath contained two bottles, and the metabolism of two rats was determined simultaneously.

After removal from the bath the bottle was wiped dry before weighing. When the bath was used at the lower temperatures the bottle, after removal, was brought to room temperature with the aid of warm water to facilitate drying and to prevent subsequent condensation of moisture on the surface.

The tightness of the bottle and absorption tubes was determined by frequent tests with a mercury manometer, and in addition several 8-hour blank determinations which included weighing the bottle and U-tubes as in an ordinary experiment were made during a wide range of temperatures. All blank tests were negligible.

The heat and water vapor produced were determined at each temperature by several 8-hour observations as shown in table 1. The chronological order of the periods of observation is also given in the table.

A total of seven male rats were used in obtaining the data though only six rats were used throughout the entire series. Thus, the fourteen 8-hour observations at 31°C. involved the use of four rats during 2 days each, and two rats during 3 days each.

The heat production of all rats was calculated to a live weight of 325 gm., this being the actual live weight of the rats at the start of the experiment. The calculation was made in accord with the two-thirds power of the live weight. The average live weight at the end of the experiment was 341 gm. No large correction of the heat production was nceessary, therefore, in any one of the series of observations.

The live weight of the rats was taken at the start of the period of metabolism measurement, 15 hours after the withdrawal of feed. The rats were 21 weeks old at the start of the experiment and 38 weeks old at the end. The respiratory quotients were not corrected for the protein metabolism.

DISCUSSION OF RESULTS

No attempt was made to measure the activity of the rats. They were visible at all times through a glass window in the cover of the bath and were remarkably quiet at all temperatures. Schwabe and associates ('38) have shown that activity is without effect on the respiratory quotient.

The most significant measure available regarding the variability of results is presented in table 1. The average of the coefficients of variability of the heat production is 4.52%, the

average probable error of the nine series being ± 0.016 Cals. It is evident, therefore, from the concordant results at a given temperature, that the activity was relatively constant and that this factor contributed but little to the variability of the results.

The correct values for the heat production at all temperatures may be somewhat higher than as observed during rest, though the long periods of observation in the present experiment seem to compensate for the lack of a record of activity. We therefore agree with Benedict and MacLeod ('29 a) that, "in practically all cases the graphic record of activity can be dispensed with, especially if it is possible to observe the animal continually." It was observed that at 33°C. and at 35°C. the rats were not only quiet but were sprawled out in an apparent attempt to expose as much surface as possible to the air. After exposure to a temperature of 35°C. for 8 hours the rats appeared to be as wet as though dipped in water.

It has been pointed out by several investigators that the height of the heat production may be affected by the temperature to which the animals have been exposed prior to the time of actual heat measurement. Schwabe and associates ('38) found that exposure to low environmental temperatures (7.8°C. to 12.2°C.) for a period of 60 days prior to the measurement of the fasting metabolism at 29°C. resulted in an increase of about 12% in heat production. In the work reported by Males and Margaz ('29) the very low temperatures to which the rats were exposed prior to the metabolism measurements may have affected their results. The temperature to which the rats used in the present study were exposed when not on experiment and while receiving feed was about 25°C., which presumably did not affect the results obtained. This temperature (25°C.) corresponds closely to that prevailing in other laboratories and seems, therefore, to represent conditions ordinarily encountered. The rats were kept in individual cages in a room containing a rat colony which

has been in a strong, healthy condition during a period of 10 years.

The small though consistent rise in respiratory quotient with rise in environmental temperature indicates that the temperature exerts a qualitative as well as a quantitative effect on the metabolism. Short exposures to cold with human subjects accompanied by shivering have no effect on the respiratory quotient (Swift, '32). However, Kayser and Dell ('37) and Kayser ('37) working with rats, guinea pigs and pigeons have shown that the increase in metabolism due to exposure to cold which occurs before the point of shivering is reached is entirely due to the oxidation of fat, even when an abundance of carbohydrate is available. In this experiment shivering did not occur and the contention of the above authors is apparently verified in view of the fact that a statistical study shows that the odds are millions to 1 that the respiratory quotient at 30°C. is greater than at 25°C. Though the computation of the respiratory quotient of the 'extra metabolism' involves unavoidable magnification of error, it seems significant that this value for the low temperatures is 0.68 whereas for the temperature 35°C. it is 0.85.

It may be remarked, in passing, that the use of a lower caloric value for CO_2 with rise in respiratory quotient exerts only a negligible effect on the computed heat production. Respiratory quotients similar to those obtained in this work at 28 to 30°C. (about 0.753) have been reported by various other workers. Wesson ('31) found a respiratory quotient of 0.769 with rats fasted 24 hours, while Schwabe and associates ('38) report an average value of 0.755 representing some 200 animals.

The data in table 1 and the graphic presentation in figure 2 indicate with apparent certainty that the critical temperature of the fasting albino rat is approximately 30°C. There was the possibility that the repeated fasts produced a lowered basal metabolism, even though the total length of each fast was only 23 hours, and such fasts occurred on an average only about twice a week. As shown in table 1 the series of four-

teen observations at 31°C. directly followed the series at 28°C., so there was very little likelihood that the lesser heat production at 31°C., as compared to that at 28°C., was due to an accumulative effect of underfeeding.

However, since 28°C. has been generally accepted as the critical temperature, special effort was made to establish this particular point beyond possibility of doubt. In order then to prevent misinterpretation due to possible influence of

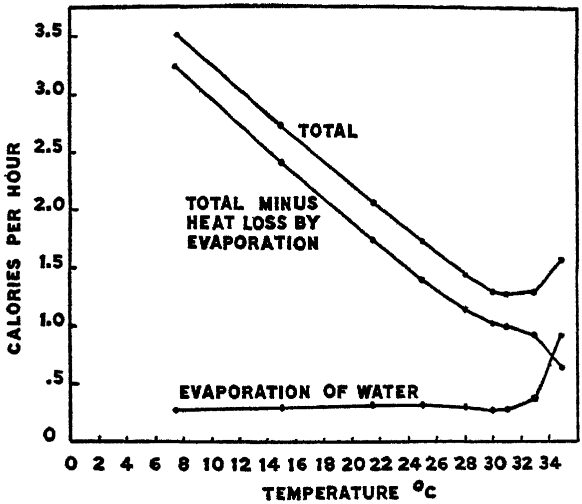

Fig. 2 The character of the heat disposal of the fasting albino rat at environmental temperatures ranging from 7.5° to 35°C.

previous fasting on the value obtained as the critical temperature and to verify the finding that the minimum heat production does not occur at 28°C., eight colony rats, never before fasted, were used in determining the metabolism at 31°C., a point within the range of thermal neutrality and, subsequently, at 28°C. Thus the order of experimentation of the first series was reversed to avoid the possibility of a lowered metabolism at 31°C. due to previous fasting. The average live weight of these rats was 297 gm. The hourly heat production was computed to a live weight basis of 325 gm., as were the corresponding values in table 1. The individual data

TABLE 1

Total heat produced and water vaporized with change in environmental temperature

ORDER OF EXPERIMENTS	NUMBER OF 8-HOUR OBSERVATIONS	TEMPERATURE	RESPIRATORY QUOTIENT		TOTAL HEAT PER HOUR			HEAT ELIMINATED BY VAPORIZATION OF WATER	
			Average	Coefficient of variation	Average	Coefficient of variation	Odds of significance[1]	Per cent of total	Coefficient of variation
		°C.		%	Cal.	%		%	%
1	8	7.5	0.710	0.8	3.51	2.8		7.9	4.9
2	11	15.0	0.723	0.8	2.72	3.7	Billions to 1	10.9	8.1
3	15	21.5	0.729	1.1	2.08	3.1	Billions to 1	15.5	8.0
4	13	25.0	0.740	1.3	1.72	3.9	Billions to 1	18.7	8.0
5	15	28.0	0.747	1.3	1.45	4.6	Billions to 1	20.5	9.2
8	11	30.0	0.759	1.0	1.30	6.2	Millions to 1	20.8	4.2
6	14	31.0	0.759	1.4	1.27	4.8	2 to 1	22.2	6.0
7	13	33.0	0.763	1.5	1.29	5.1	1 to 1	27.8	10.0
9	6	35.0	0.775	1.1	1.57	6.6	1 billion to 1	59.2	13.1

[1] These odds represent the significance of the difference between each heat production value and the one at the preceding lower temperature.

are given in table 2. The evidence is again overwhelming that fasting rats have a higher heat production at 28°C. than at 31°C.

These rats were 15 weeks of age while those of the first series (table 1) were about 32 weeks old when used at these temperatures. Though this experiment was not designed to show the effect of age on basal metabolism, the higher level of heat production of the younger rats at both temperatures indicates that metabolism decreases with age—in contrast to the observations of Benedict and MacLeod ('29). However, our results are concerned with two groups of rats at ages of

TABLE 2

Heat production at an environmental temperature of 31°C. compared with 28°C.

RAT NUMBER	HEAT PRODUCTION PER HOUR		HEAT LOST BY EVAPORATION OF WATER	
	28°C.	31°C.	28°C.	31°C.
	Cal.	*Cal.*	%	%
67	1.48	1.41	18.7	24.4
68	1.52	1.41	16.6	19.8
69	1.56	1.44	18.8	22.7
70	1.71	1.49	23.6	25.1
71	1.49	1.36	18.7	23.5
72	1.67	1.47	18.8	26.3
73	1.58	1.48	19.5	22.4
74	1.63	1.45	23.2	26.8
Av.	1.58	1.44	19.7	23.9

about 3 and 7½ months of age. Black ('29) has reported a higher basal metabolism in very old rats (700 days) than in younger ones (7 to 8 months).

It may be observed from table 1 that although the percentage of heat eliminated by vaporization of water increases steadily with rise in environmental temperature, the amount of heat thus eliminated is essentially constant over an extended range of temperatures and does not increase materially until shortly before the point of hyperthermal rise is reached.

SUMMARY

The heat production of the albino rat in the postabsorptive state was determined in 122 8-hour periods of measurement

at environmental temperatures ranging from 7.5° to 35.0°C. The critical temperature for the fasting rat was found to be 30°C. The metabolism was constant over a range of temperatures extending from 30°C. to 33°C. The percentage of the total heat eliminated as latent heat of water vapor increased steadily from 7.9% to 59.2%, with rise in environmental temperature from 7.5°C. to 35°C., though the absolute amount of heat eliminated in this manner was practically constant up to 31°C., above which a definite increase took place.

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EFFECT OF SEVERAL CALCIUM SALTS ON THE UTILIZATION OF LACTOSE [1]

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TWO FIGURES

(Received for publication May 27, 1939)

The relationship of lactose and calcium metabolism has long been recognized, especially the role of lactose in promoting better absorption of calcium from the intestinal tract. The utilization or retention of calcium has been studied after oral administration of different forms of calcium and as influenced by different forms of carbohydrate in the ration, but little attention has been given to the possible influence of the various calcium salts on the utilization of other dietary constituents. The present observations in this field were incident to experiments being conducted in this laboratory on the cataractogenic action of lactose as influenced by inorganic constituents of the ration. The calcium salts used were those most commonly employed in present day calcium therapy. Observations in this laboratory indicate considerable variation in the effect of different calcium salts on the digestion or absorption of lactose in rats.

EXPERIMENTAL

All animals were started on experimental rations at between 25 and 30 days of age and were so distributed as to afford adequate litter-mate controls. The basal rations used were complete in the usual sense, i.e., in quantity and proportion of

[1] Published as contribution no. 347 of the Massachusetts Agricultural Experiment Station.

ingredients and in vitamin supplements. They varied only in the type of carbohydrate incorporated in the ration and in the calcium salts used as supplements. All rations were fed ad libitum with accurate records of food intake. The composition and designation of the specific rations were as follows:

TABLE 1

Experimental rations

Composition	*60% lactose*	*65% starch*	*65% other CHO*	*25% galactose*
Lactose	60			
Starch (corn)	5	65		40
Dextrin				
Sucrose			65	
Glucose				
Galactose				25
Casein (technical)	15	15	15	15
Crisco	9	9	9	9
Cod liver oil	2	2	2	2
Salt mixture (O & M)	4	4	4	4
Yeast (dry brewers)	5	5	5	5

Calcium salts were added to these rations in quantities calculated to supply respectively 1.0 or 0.5% of calcium in addition to the 0.5% already present as part of the incorporated salt mixture. The calcium salts used were the carbonate, tricalcium phosphate, citrate, lactate, levulinate and gluconate, and were of C.P. or U.S.P. grade. Since lactose was the carbohydrate of primary interest in this investigation, by far the most data were accumulated with rats on the 60% lactose ration with and without the added salts. Starch, dextrin, sucrose, glucose and galactose rations plus added calcium salts served as controls.

In the first series of experiments, 1.0% of calcium was added to the basal ration as follows: 2.6 gm. tricalcium phosphate, 2.5 gm. calcium carbonate, 8.0 gm. (later reduced to 6.0 gm.) calcium lactate, 11.2 gm. calcium gluconate respectively per 100 gm. of ration. Careful record was kept of survival, growth, food intake, evidence of diarrhea, incidence and rate of cataract development and blood sugar level during absorption. All experiments ran for 9 weeks or longer.

The results of feeding the various calcium salts incorporated in a lactose ration are shown in the table (tablė 2). The growth, survival and cataract development of rats receiving the carbonate or phosphate approximated those on the basal lactose ration, and the diarrhea was somewhat less. Blood sugar tended to be slightly higher in rats receiving the carbonate or phosphate (150 mg. and 146 mg. per 100 cc.), respectively as compared with litter-mate controls on the 60% lactose ration (135 mg. per 100 cc.). The rats receiving the calcium lactate or gluconate showed definite toxic symptoms, retarded growth, poor survival and moderate to severe diarrhea in the case of the gluconate. More striking still was the lower blood sugar (103 mg. per 100 cc.) and absence of lenticular changes in rats on the gluconate in contrast to high blood sugar and usual incidence of cataract in all other groups on lactose rations. Collectively, these findings would indicate that the gluconate, and to a lesser extent the lactate, interfere in some manner with the utilization of lactose.

Calcium salts incorporated in starch, dextrin, sucrose, galactose and glucose rations at the 1.0% level were well tolerated by rats with no untoward effects. The good growth and general well-being of rats on these five carbohydrates were evidence that calcium salts as such, at the levels fed, were not seriously toxic or detrimental, nor were there any significant differences in the effect of individual salts (fig. 1). Growth efficiency calculated from the total weight gained in 9 weeks divided by the total food eaten in the same period was remarkably constant for the different sugars, irrespective of the calcium salts added. Since expense necessitated the termination of galactose experiments when cataract developed, growth efficiency in this group was based upon data for a shorter period but showed no variation with added calcium salts. The rapid and constant time for development of cataract on 25% galactose rations with or without added calcium salts is further evidence that absorption of this single sugar was not inhibited. Previous work by Mitchell et al.

TABLE 2

Effect of calcium salts on the utilization of lactose

RATION	NUMBER OF RATS	SURVIVAL PER CENT	GAIN IN WEIGHT IN 9 WEEKS		SEVERITY OF DIARRHEA EARLY — LATE	INCIDENCE OF CATARACT		BLOOD SUGAR	
			Male	Female		Early changes	Mature cataract	Milligrams per 100 cc.	Number of determinations
			gm.	*gm.*		%	%		
1.0% calcium in added salts									
60% Lactose	32	94	111	92	+++—+	100	37	135	(82)
60% L — 2.6% $Ca_3(PO_4)_2$	33	96	96	85	++—0	100	42	150	(80)
60% L — 2.5% $CaCO_3$	14	100	73	75	+—0	100	39	146	(53)
60% L — 8.0% Ca lactate	4	0	..	..	+++	..	..	..	..
60% L — 6.0% Ca lactate	4	50	9	10	++—0	100	0		
60% L — 11.2% Ca gluconate	13	55	23	6	++++—++	0	0	103	(16)
70% Starch	15	100	145	111	0 0	0	0	113	(37)
0.5% calcium in added calcium salts									
60% Lactose	15	100	124	105	++—0	100	53	145	(29)
60% L — 1.3% $Ca_3(PO_4)_2$	6	100	112	67	+—0	100	8[1]	146	(10)
60% L — 2.4% Ca citrate	6	100	125	81	++—0	100	20[1]	156	(9)
60% L — 4.0% Ca lactate	9	100	81	79	+—0	100	45	151	(13)
60% L — 3.4% Ca levulinate	4	100	100	84	+—0	100	25[1]	127	(11)
60% L — 5.6% Ca gluconate	18	67	52	38	+++—++	22	0	100	(21)
60% L — 5.6% Na gluconate	8	38	104	113	++++—+++	0	0	106	(6)
65% Starch	11	100	202	122	0 0	0	0	113	(14)
65% S — 5.6% Ca gluconate	7	100	182	128	0 0	0	0	117	(11)
65% S — 5.6% Na gluconate	2	100	190	173	0 0	0	0	131	(6)

[1] Rats from cataract-resistant litters are included in these averages.

('37) indicates that lens injury correlates with high blood sugar (fig. 2).

In the second series of experiments, 0.5% of added calcium was given as 1.3 gm. tricalcium phosphate, 2.4 gm. citrate, 4.0 gm. lactate, 3.4 gm. levulinate and 5.6 gm. gluconate, per 100 gm. of food. The same type of observations was made as in the first series, plus a few bone ash determinations. (The percentage of ash in the dry fat-free bone was practically constant (68.4 to 70.2) for all rats, irrespective of the

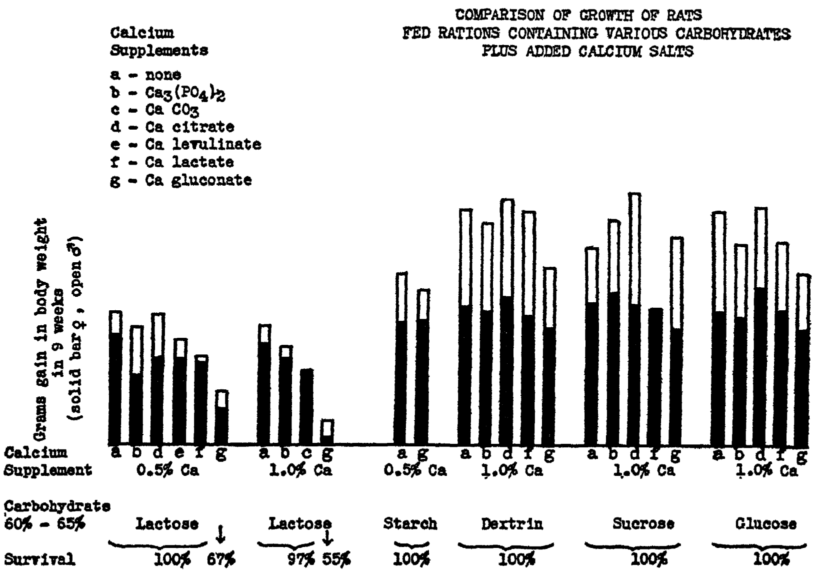

Figure 1

ration, and served to indicate that the calcium metabolism per se was not disturbed.) The results of the two series of experiments are similar, the second serving to verify the first but obviously modified because the salts were added at half the previous level to reduce mortality and allow more prolonged study of the metabolic abnormalities (table 2).

Again certain calcium salts, namely the phosphate, citrate and levulinate exerted no appreciable effect as evidenced by rate of growth, growth efficiency, survival, blood sugar level

or cataract development. The lactate at the lower 4.0 gm. level was not so toxic as in the first series, although growth was slightly retarded. Growth efficiency is somewhat lower on all lactose rations than on other carbohydrate but is not altered by the above salts except slightly in the case of the lactate.

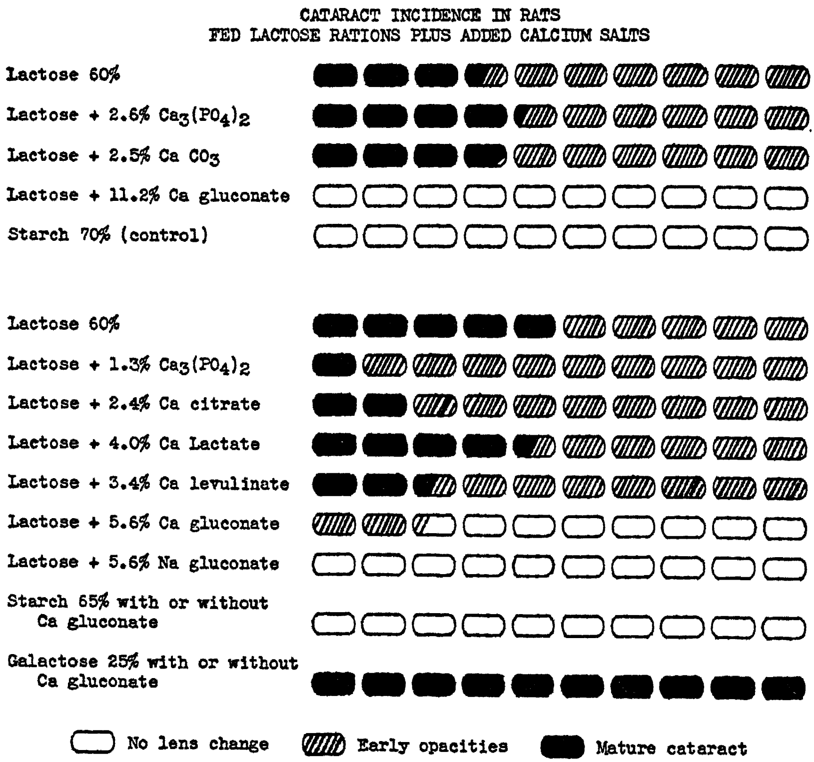

Figure 2

The calcium gluconate, however, still exerted its inhibitory effect when fed at the lower level (0.5% of calcium), and results were similar for three different brands.[2] Retarded growth and lowered growth efficiency, poor survival, severe

[2] The calcium gluconate was obtained from three different commercial sources: 1. Sandoz Chemical Works, Inc.; 2. Chas. Pfizer and Co., Inc.; 3. Pfanstiehl Chemical Company.

diarrhea, reduced blood sugar and slight if any lenticular changes indicated that the lactose was not being assimilated normally (fig. 2). The fact that both glucose and galactose fed as such resulted in as good growth efficiency in presence of the gluconate as without it indicates no interference with absorption of the simple sugars. The enzymatic hydrolysis of starch, dextrin and sucrose also appear to proceed normally. The failure of the blood sugar to show the usual rise during absorption when calcium gluconate was associated with lactose seemed to indicate that the calcium gluconate inhibited the enzymatic hydrolysis of the lactose. The severe diarrhea is also indicative of an excess of lactose remaining, as such, in the alimentary canal. In spite of the fact that calcium gluconate is one of the salts most widely recommenended for therapeutic use,[3] some indication of unfavorable reactions have previously been mentioned. Johnson ('35) found that calcium gluconate inhibited glucose absorption from the intestine of his experimental dogs. There is also recognition of the fact that large doses of either the lactate or the gluconate used therapeutically may cause diarrhea (correspondence, '38). In our own experience urinary calculi have been found in several of the rats fed on calcium gluconate and never in rats fed the other calcium salts.

References have been made to the severity of the diarrhea on the various lactose rations. It is well known that young rats fed a ration containing 50% or more of lactose tend to develop diarrhea, more severe in some than in others, but that they usually develop a tolerance or outgrow this tendency after a few weeks. An abrupt shift from a stock ration to a high level of lactose is frequently fatal to young rats. For this reason, a graduated proportion of a starch ration was mixed with the lactose ration for a few days at the beginning of these experiments in order to keep the rats alive until they developed some degree of tolerance. It has been notable throughout this experiment that most of the rats fed either of

[3] New and Nonofficial Remedies, 1938, p. 158. Useful Drugs, 1936, p. 69. Epitome of U. S. Pharmocopeia and National Formulary, 1937, p. 48.

the gluconate salts in a lactose ration continued to show almost as severe diarrhea after several weeks as at first; a few on calcium gluconate developed a degree of tolerance.

Since no other calcium salts investigated approached the gluconate in toxic or inhibitory action, the gluconate radical was brought under suspicion. Consequently, sodium gluconate was fed at a comparable level incorporated in a 60% lactose ration. The untoward effects were strikingly similar but rather more severe than with the calcium gluconate fed to litter-mate controls, i.e., the survival was poorer, diarrhea worse and lens changes absent, but in the few rats which did survive the rate of growth and growth efficiency improved after a few weeks in spite of a persistent diarrhea, a finding which cannot be readily explained. Thus it would appear that the gluconate radical, rather than the calcium, must be the part which intereferes with lactose activity in the alimentary canal. One may postulate some such phenomenon as 'competitive inhibition,' in enzyme action, due to similarity in structure of the lactose and the gluconate radicals. In vitro work on this phase of the subject is in progress but is not yet conclusive.

SUMMARY

1. Rats were fed an adequate ration containing 60% lactose, plus 1.0 or 0.5% of calcium added in the form of six different calcium salts, respectively, tricalcium phosphate, carbonate, citrate, lactate, levulinate and gluconate.

2. With the phosphate, carbonate, citrate and levulinate rats showed as good growth, less diarrhea in general, the same degree of galactemia and a similar incidence of cataract as on the plain 60% lactose ration. Calcium lactate was somewhat toxic at the higher level but at the lower level results were more nearly comparable to those with the other calcium salts mentioned above.

3. Calcium gluconate fed at the 1.0% calcium level resulted in poor survival, loss of weight, severe diarrhea and low normal blood sugar. At the 0.5% level of calcium survival

was better, but growth was poor, diarrhea moderate to severe, blood sugar a low normal and lenticular changes few if any.

4. Calcium gluconate exerted no apparent inhibitory effect upon the absorption or utilization of starch, dextrin, sucrose, glucose or galactose.

5. Sodium gluconate exerted a deleterious effect similar to but more severe than calcium gluconate when fed in a lactose ration.

6. These criteria indicate that the gluconate radical may interefere with lactase activity in the intestinal tract. The phenomenon of 'competitive inhibition' in enzyme action is postulated as a possible explanation of these findings.

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FATTY LIVERS AS A RESULT OF THIAMIN ADMINISTRATION IN VITAMIN B_1 DEFICIENCY OF THE RAT AND THE CHICK [1, 2]

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FOUR FIGURES

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INTRODUCTION

While investigating the pathology of an uncomplicated vitamin B_1 deficiency in the rat it was discovered ('38) that if the animals were restored to normal by the addition of thiamin to the vitamin B_1 deficient ration a hydropic degeneration appeared in the liver cells. Rats suffering from vitamin B_1 deficiency, if restored to normal by feeding them a ration rich in fat and free of carbohydrate, had retained a normal liver cell structure. These observations led to more thorough histologic and chemical studies concerning the effect of thiamin therapy in B_1 avitaminosis. Experiments were carried out with growing rats and with growing chicks.

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

[2] These studies were aided in part by a grant from the Wisconsin Alumni Research Foundation and in part by the Works Progress Administration.

EXPERIMENTAL

Part I. Studies on the rat

Ration 112 of Arnold and Elvehjem ('38 a) was used to produce vitamin B_1 deficiency. Its composition was as follows:

Sucrose	62
Purified casein	18
Autoclaved peanuts	10
Autoclaved A.B. yeast	4
Salts I	4
Liver extract Factor W concentrate ⇌	2 liver extract
Percomorph oil	3 drops weekly

An experiment was first made to determine the protective ability of various agents against the fatty livers which result when rats are relieved of vitamin B_1 deficient symptoms by thiamin supplements. Litter mate weanling albino rats were depleted of their vitamin B_1 stores. When severe deficiency symptoms were apparent, two animals which served as controls were removed for study. The remaining animals were removed after having received various supplements over a 1-week period. The dietary supplements and the results of this experiment are summarized in table 1.

The livers were examined microscopically with the use of H and E stain as well as with osmic acid (Romeis, '32) for the detection of fat. Occasionally pancreas, kidney and adrenal gland tissue was also examined.

After histologic specimens had been obtained the remainder of the livers was weighed, dried at 100° C. and then re-weighed. The difference in weight represented moisture. Lipids were determined by grinding the dried samples and extracting for 18 hours with ethyl ether.

Supplements of thiamin resulted in an increase in liver lipids as is shown in table 1. An increase in liver size and weight also resulted from thiamin therapy. The increase in liver weight could not be completely accounted for from the rise in lipids. Histologic observations substantiated the chemical data since all cases which had received thiamin

showed hydropic degeneration and fat metamorphosis in the liver. At autopsy these livers were observed to be enlarged and light in color. The control animals (vitamin B_1 deficient) had a normal liver cell structure. It is obvious that none of the supplements tested were protective against the fatty liver condition.

Since an inanition accompanies severe vitamin B_1 deficiency, it seemed advisable to test the effect of thiamin on liver fat in starvation. To this end four litter mate weanling rats were

TABLE 1

The effect of thiamin administration upon liver structure and composition in the B_1 deficient rat

LOT	NUMBER OF ANIMALS	DIETARY SUPPLEMENTS	LIVER PER CENT OF BODY WEIGHT	LIPIDS	MOISTURE	DEGREE OF HISTOLOGIC DAMAGE
				%	%	
I	2	None	3.00	6.46	73.2	0
			3.05	7.28	70.4	0
II	2	100 µg. thiamin/100 gm. of	5.25	9.13	73.3	+++
		ration	6.00	9.30	70.6	+++
III	2	100 µg. thiamin/100 gm. +	6.10	10.35	75.6	++
		lipocaic = 10% fresh pancreas	5.50	9.28	70.1	+++
IV	2	100 µg. thiamin/100 gm. +	5.72	8.33	71.1	+++
		5 mg. choline/rat/day	5.53	8.38	69.0	+++
V	2	Isocaloric replacement of carbo-				
		hydrate with fat + 20 µg.	5.88	12.22	68.3	++
		thiamin/100 gm.	4.99	12.30	70.6	++

starved. Two were injected intraperitoneally with 100 µg. of thiamin on the third day of starvation. All animals were removed on the fourth day. Microscopic examination of the livers revealed only a slight fat reaction in the cells near the central veins in all these cases. These observations suggest that the inanition which usually accompanies severe B_1 avitaminosis is not concerned with the fatty livers resulting from thiamin therapy.

It has been suggested by Sure ('35) that an antagonistic relationship exists between thiamin and thyroxin. The effect

of the latter on the fatty livers obtained with thiamin therapy in B_1 avitaminosis was next studied. Four young rats received 5 mg. of desiccated thyroid daily after having been kept on ration 112 for 4 weeks. This supplement produced severe B_1 deficiency symptoms before the end of the fifth week. Two of these animals were then removed while the remaining two were removed 24 hours after each had received 400 μg. of thiamin intraperitoneally.

H and E stained sections, as well as osmicated sections revealed a normal liver cell structure in all cases. These results indicate that the increased rate of metabolism induced by desiccated thyroid feeding was responsible for the prevention of the fatty liver condition occurring in thiamin therapy.

Part II. Studies on the chick

To determine if this hitherto unnoticed liver pathology could be produced in other species, studies were next made on the chick. Vitamin B_1 deficiency was produced by feeding 1-day-old chicks ration 242A of Arnold and Elvehjem ('38 b). This ration consists of:

A. Autoclaved portion		*B. Untreated portion*	
Ground yellow corn	57	Dried whole liver	1
Wheat middlings	25	Iodized salt	1
Crude acid precipitated casein	12	$CaCO_3$	1
		$Ca_3(PO_4)_2$	1
		Cod liver oil	2

In a preliminary experiment with six chicks it was shown that thiamin, when given to vitamin B_1 deficient chicks over a period of 7 days in daily oral doses of 50 μg., produced enlarged light colored livers. Microscopic examination showed that fatty metamorphosis was present.

In the next experiment an attempt was again made to determine the protective value of various agents. The experimental procedure was as follows:

Lot	*Number of animals*	*Dietary regimen*
I	4	Basal ration + 0.05% desiccated thyroid
II	4	Basal + 5 mg. choline/chick/day
III	4	Basal + 30% acid precipitated casein

Two of the birds from each lot served as controls and were removed when severely vitamin B_1 deficient. The remaining birds were given intraperitoneal injections of 400 µg. of thiamin when in the severely deficient state and were sacrificed 48 hours later.

Previous results obtained with the rat were substantiated since no liver pathology resulted in the birds receiving both desiccated thyroid and thiamin. Neither choline nor a high level of casein in the diet protected the livers of these birds against fatty metamorphosis. All the controls (no thiamin) had normal livers.

TABLE 2

The effect of thiamin (or co-carboxylase) on liver glycogen in polyneuritic chicks

CHICK	DIETARY SUPPLEMENTS	DAYS ON THIAMIN THERAPY	PER CENT GLYCOGEN	DEGREE OF HISTOLOGIC DAMAGE
1225	20 µg. thiamin + 5 mg. choline	2	2.23	+
1227	200 µg. thiamin + 5 mg. choline	2	9.81	++
1228	200 µg. thiamin + 5 mg. choline	2	2.63	+++
1230	200 µg. thiamin + 5 mg. choline	2	8.86	+++
1231	400 µg. thiamin + 5 mg. glucose	1	3.14	+++
1235	500 µg. co-carboxylase	4	7.82	+++
1317	200 µg. thiamin + 0.08 units insulin	1	10.13	+++
1319	200 µg. thiamin + 0.08 units insulin	1	9.97	+++
(7 chicks)	(B_1 deficient, no supplement)	..	Av. 0.90	0

Further chemical studies were made on the fatty livers resulting from thiamin therapy in polyneuritic chicks in an attempt further to elucidate the nature of the structural changes. Table 2 presents some typical results of experiments in which it was attempted to correlate liver glycogen content with histologic observations. In these studies the dietary supplements were injected intraperitoneally. The birds were killed by decapitation and portions of liver immediately homogenized in alcoholic KOH solution for glycogen determination (modified Kerr method, '36).

It is apparent that a marked increase in liver glycogen occurs with thiamin therapy in polyneuritic birds. It is also obvious that insulin seemed to increase the liver glycogen de-

position when given along with thiamin and that it did not protect against fatty metamorphosis. Co-carboxylase was as effective as thiamin in producing the typical fatty livers.

The severity of the pathologic changes could not be correlated with the glycogen storage since livers with 3 to 4% glycogen were as severely affected as those with 8 to 10% glycogen.

In subsequent studies more complete chemical analyses were made. Some typical results are presented in table 3. Since in previous experiments the birds had been continued on the basal diet after thiamin therapy, it was also of interest

TABLE 3

The effect of thiamin on average liver composition

NUMBER OF BIRDS	RATION	LIVER PER CENT OF BODY WEIGHT	MOISTURE	EtOH EXTRACT	MG. P IN EtOH EXTRACT PER GRAM DRY LIVER (PHOSPHOLIPID P)	PROTEIN
			%	%		%
12	242A + 300 μg. thiamin	3.91	71.70	8.67	3.43	12.20
8	242A only	2.50	73.20	7.44	5.03	17.80
5	242A + 300 μg. thiamin [1]	2.17	73.70	8.32	4.48	17.50

[1] Autopsied in starvation 24 hours after the administration of thiamin.

to see if the typical enlarged fatty livers would result if the polyneuritic birds were starved after thiamin therapy. Typical polyneuritis was produced as usual. The thiamin was injected intraperitoneally 24 hours before autopsy.

At autopsy liver samples were preserved for histologic study. The remaining liver tissue was dried at 100°C. for moisture determination. The dried samples were then extracted six times with hot ethyl alcohol. Phosphorus was determined on aliquots of this extract and is represented as phospholipid phosphorus. The protein was calculated from the nitrogen in the residue after alcohol extraction.

When calculated on a percentage basis, the fat content in the livers of thiamin treated birds was only slightly higher than that of the controls. However, since the livers of birds

receiving thiamin plus basal ration were hypertrophied, it is obvious that the total fat in the livers of these birds was markedly increased over that of the controls. Similarly the total moisture was greatly increased with thiamin therapy. On the other hand, the total protein and phospholipid values compare favorably with the controls although they are lowered in

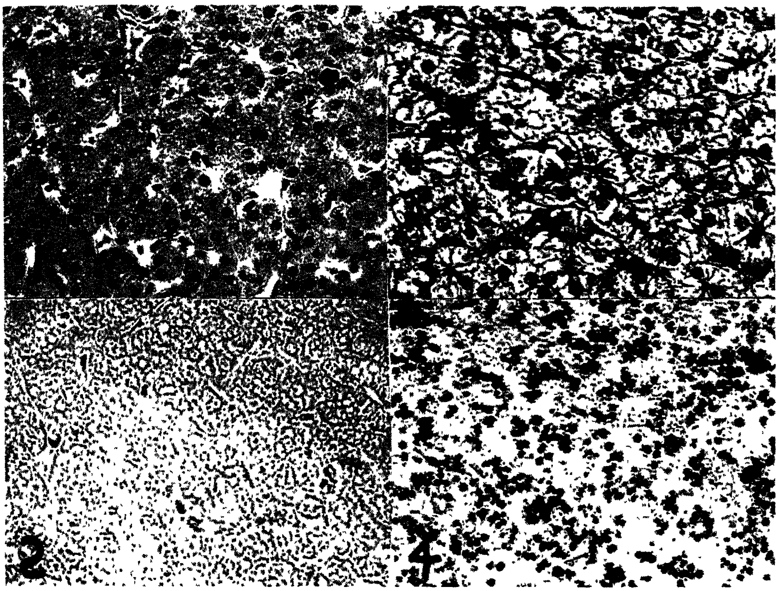

Fig. 1 Chick no. 1407. Vitamin B_1 deficient. Normal liver cell structure. H. and E. stain. $\times$ 440.

Fig. 2 Chick no. 1407. (Same as figure 1.) No fat staining. Maximov's osmic acid stain. $\times$ 440.

Fig. 3 Chick 1404. Vitamin B_1 deficient chick injected with 300 μg. of thiamin 24 hours before autopsy. Hydropic degeneration. H. and E. stain. $\times$ 440.

Fig. 4 Chick 1404. (Same as figure 3.) Fatty metamorphosis. Maximov's osmic acid stain. $\times$ 440.

the thiamin treated birds when calculated on a percentage basis. Similar phospholipid changes were observed by Flock ('39) who studied fatty livers in geese. From these data it would seem that the abnormal liver composition induced by thiamin therapy is largely due to storage of exogenous food

substances since the group which had received thiamin and then had been starved for 24 hours had a liver composition comparable to the controls. However, in this group there was also a slight increase in the per cent of liver fat.

Histologic studies on these livers revealed that the birds receiving the basal ration only had normal livers (figs. 1, 2). Typical hydropic and fatty liver changes were present in the two groups receiving thiamin (figs. 3, 4). However, the changes were much less severe in the group which had not had access to food after thiamin therapy.

DISCUSSION

The histologic changes which appeared in the livers as the results of thiamin therapy in vitamin B_1 deficient animals are of interest since this vitamin is rather extensively used clinically. Cowgill ('38) and others have used thiamin at rather high levels and failed to observe any adverse effects. Cowgill states that no toxic symptoms were observed in doses 25,000 or more times the estimated daily human requirement. Recently Steinberg ('38) has observed Herpes Zoster resulting in patients treated with large doses of thiamin. He suggested that thiamin in large doses irritates peripheral nerve plates. With few exceptions, reported data would suggest a wide range of tolerance of this vitamin by the animal body. Doses of the vitamin used in the studies reported here are considerably below toxic levels as suggested by Molitar and Sampson ('36) and by Hecht and Wiese ('37). It is true that no gross manifestations of toxicity were noticed in the present study. An increased liver size and occasionally a slight atrophy of the pancreas were the only abnormalities noted at autopsy.

The results obtained in these studies are in agreement with Whipple and Church ('36) and McHenry ('37) who have also demonstrated that thiamin increases liver fat. The protective value of choline against liver fat accumulation as reported by McHenry could not be confirmed when choline was fed at a level of 5 mg. daily.

Whether the extreme histologic reactions in the liver resulting from thiamin therapy in B_1 avitaminosis would lead to permanent liver damage is still a question. Recent evidence with the chick indicates that there is a gradual resorption of the liver fat over a period of 6 weeks, even though thiamin is administered continuously. The fact that the animals made remarkable weight gains and showed no gross ill effects from the thiamin therapy would indicate that even though the liver tissue appears abnormal histologically, its function has not been seriously and permanently disturbed.

SUMMARY AND CONCLUSIONS

When thiamin was administered to vitamin B_1 deficient chicks or rats hydropic degeneration and fatty metamorphosis occurred in the parenchyma of the liver cells. This histologic reaction in the liver was not prevented by choline, lipocaic or by diets high in fat or in casein. Desiccated thyroid was effective in preventing the histologic reactions in the livers.

Chemical analyses on these livers showed that there was an increase in total fat, glycogen and moisture. The phospholipid and protein remained unchanged.

Thiamin therapy in experimental vitamin B_1 deficiency causes an excessive production of free fat in the liver cell which disrupts the normal cell structure.

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FACTORS INFLUENCING STORAGE OF PROTEIN WITH LOW-CALORIE DIETS

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TWO FIGURES

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The purpose of this report is to demonstrate a) the effects of different forms of low-calorie diets upon the metabolism of protein, and b) the protein-sparing action of between-meal feedings of carbohydrates with low-calorie diets of the same composition with respect to carbohydrate and fat, but different amounts of animal and vegetable proteins. The importance of knowledge of the different factors which may influence the metabolism of protein with low-calorie diets is obvious from the importance of protein in nutrition and from the many people who, for different reasons in health and in disease, must reduce their intake of food. Low wage incomes and obesity are examples in health; diabetes is an example of disease.

Haggard and Greenberg ('35) have shown that between-meal feedings increase the net muscular efficiency of the human body and that the increase is associated with an increase of carbohydrate metabolism. The observations in general fitted in with the well-known fact that mechanical work is best done with carbohydrates; it is done less efficiently with fats and least efficiently with protein. This, however, is not the only beneficial effect of between-meal feedings. The latter prevent the stomach from becoming empty and an empty

stomach may impair efficiency, regardless of an adequate caloric intake by production of fatigue, etc.—Carlson's accessory phenomena of hunger.

In general, urinary excretion of nitrogen is governed by the intensity of the amino acid metabolism and, in man, the metabolism of tissue protein is never in abeyance (Borsook and Keighley, '35). Even with nitrogen equilibrium, endogenous (tissue) protein was found to account for more than half of the total urinary nitrogen (Beard, '35). In the construction of low-calorie diets, consideration must, therefore, be given to the conditions which tend to conserve nitrogen and one of these is the intake of protein, quantitatively and qualitatively; retention of protein is more readily accomplished with liberal than with low-protein diets and, with the same intake of protein, more readily accomplished with animal than with vegetable proteins. It is also a well-known fact that the total amount of carbohydrate in the diet also influences the degree of protein storage (Lusk, '28; Sherman, '37). Recently, however, Larson and Chaikoff ('37) demonstrated another variable in the protein-sparing action of carbohydrates, namely, time. These authors have shown that carbohydrates exert their maximum protein-sparing action only when they are available at the time that the intensity of the protein metabolism is at its maximum and that the interval during which extra carbohydrate is able to exert any nitrogen-sparing action is limited to about 4 hours before and 4 hours after the ingestion of the regular meal. The findings, in general, fit in with the more recent observation of Cuthbertson and Munro ('39) that, regardless of the amount of carbohydrate and protein fed, whether the nitrogen balance was positive or negative depended upon the number of meals per day.

The data to be reported here are based upon experiences with eight different diets of diabetics during the last 20 years. As will be shown, they afford additional proof of the influence of time in the protein-sparing action of carbohydrates and thus the importance of between-meal feedings of carbo-

hydrates, in order to maintain nitrogen equilibrium in adults and permit optimum storage of protein in children on low-calorie diets. The data include 875 nitrogen determinations of 24-hour samples of urine obtained with diets of different carbohydrate, fat and protein contents divided into three meals a day only and also with diets which included between-meal feedings of different amounts of carbohydrates.

In the interpretation of the following data, it is necessary to point out that, though all of the foods were weighed accurately, their exact content of carbohydrate, fat and protein was not known, because of the variations in chemical composition of food materials. The average values shown in table 1, however, approximate the actual very closely, since they were calculated from a large number of observations. It will be noted that no nitrogen balance was calculated from less than 74 days of observation of diets divided into three meals a day nor upon less than 51 days of observation of diets divided into six meals a day. No balance was, therefore, calculated from less than 200 meals. Furthermore, it should be noted that, this was a comparative study and that there was no reason for greater variations in composition of the food materials in one diet than in another.

All nitrogen determinations of the urines were made in duplicate with the macro-Kjeldahl technic. The combined data are, briefly, summarized in table 1.

It will be noted that, though the excess excretions were not very great, the average values clearly indicate that nitrogen equilibrium was not maintained either with the modified Allen (diet 1) or with the low carbohydrate-high fat-low protein diet (diet 2). The data thus fit in with the earlier experiences of Geyelin and Du Bois ('16). With the moderate amount of carbohydrate—100 gm.—but with no reduction of the fat nor increase of the protein content (diet 3) there was much less difficulty in maintaining nitrogen equilibrium, but the average output of nitrogen still exceeded the average intake. A very definite change of the protein metabolism was, however, noted when the latter diet was replaced by the

TABLE 1

Showing relationship between type of diet and nitrogen balance in diabetes mellitus

	1	2	3	4	5	6	7	8
	MODIFIED ALLEN	LOW CARBOHYDRATE HIGH FAT	MODERATE CARBOHYDRATE HIGH FAT	HIGH CARBOHYDRATE-LOW FAT-MODERATE PROTEIN	SAME AS DIET 4 BUT WITH BETWEEN-MEAL FEEDINGS OF 10 GM. CARBOHYDRATE [1]	HIGH CARBOHYDRATE-LOW FAT-LIBERAL PROTEIN	SAME AS DIET 6 BUT WITH BETWEEN-MEAL FEEDINGS OF 10 GM. CARBOHYDRATE [1]	SAME AS DIET 7 BUT WITH BETWEEN-MEAL FEEDINGS OF 10 GM. CARBOHYDRATE [1]
Diet (average values)								
Carbohydrate	35.4	48.7	108.7	248.7	241.4	256.3	251.4	246.8
Fat	142.8	147.3	142.5	56.0	53.2	44.1	42.9	43.4
Protein	53.1	56.2	52.6	73.1	71.0	104.6	106.7	109.2
Total glucose	80.5	96.0	153.5	296.7	287.9	321.4	317.6	314.4
Total calories	1639	1745	1928	1791	1728	1840	1818	1815
Average weight (kg.)	70.4	68.3	72.5	64.8	70.6	69.8	76.1	74.4
Total protein (gm./kg.)	0.75	0.82	0.72	1.13	1.00	1.49	1.40	1.46
Animal protein								
Total (gm.)	36.3	42.1	40.8	35.8	37.2	66.0	66.9	68.4
Per cent of total	68.4	74.9	77.5	48.9	52.4	63.1	62.7	62.6
Gm./kg.	0.51	0.62	0.56	0.55	0.53	0.94	0.88	0.91
Days of observation	155	74	136	218	111	68	51	62
Nitrogen balance								
Intake	8.5	9.0	8.4	11.7	11.3	16.7	17.1	17.4
Output [2]	10.5	10.7	9.0	10.7	9.7	14.5	13.3	12.7
Retention or loss (gm.)	−2.0	−1.7	−0.6	+1.0	+1.6	+2.2	+3.8	+4.7
Retention or loss (gm./kg.)	−0.028	−0.025	−0.008	+0.015	+0.022	+0.031	+0.050	+0.063
Intake/output × 100	0.81	0.84	0.93	1.09	1.16	1.15	1.28	1.37

[1] The total food value of this diet includes the between-meal feedings.

[2] Includes allowance of 10% of nitrogen in faeces.

high carbohydrate-low fat-moderate protein diet (diet 4); the average intake now exceeded the average output. As was pointed out in the first report of this diet (Rabinowitch, '30) retention of nitrogen is one of its most striking metabolic effects. In fact, subsequent experiences (Rabinowitch, '31) showed that the lowest urinary nitrogen values ever found in man were, judging from the literature (Rabinowitch, '38 a) found with this diet.

The above experiences fit in with the well-known experiments of Kayser and of Tallquist (Sherman, '37) in which, by feeding isodynamic quantities of carbohydrate and fat, it was shown that carbohydrates tend to enhance, whereas fats tend to interfere with, storage of nitrogen in the body. A variable which must, however, be considered in the interpretation of the above data is the higher intake of protein with diet 4 than with diets 1, 2 and 3. It will be noted that, whereas diets 1, 2 and 3 contained about 50 gm. only of protein, diet 4 contained about 75 gm. The difference is, approximately, the same when the intake of protein is expressed as grams per kilogram of body weight. That the increase of protein was an important variable will be noted subsequently.

Diet 5 clearly shows the protein-sparing action of between-meal feedings of carbohydrates with diets of low caloric value. It will be noted that the total allowance of food with this diet was the same as with diet 4; but, from the total allowance, the equivalent of 10 gm. of carbohydrate was fed between meals (11.00 A.M. and 4.00 P.M.) and also before retiring. The result of this change of treatment was a still greater retention of nitrogen.

The origin of these between-meal feedings was entirely accidental, namely, an observation by one of our diabetics that he always felt better whenever he took, from his total allowance, some food (an apple or an orange) between meals. Except for the known fact that frequent feedings prevent the stomach from becoming empty and thus tend to diminish weakness and fatigue (Carlson's accessory phenomena of hunger) the explanation of the improvement of health in

general was not clear until the publication of Haggard and Greenberg on diet and physical efficiency ('35). That these between-meal feedings have approximately the same effect upon the respiratory quotient in diabetics as in non-diabetics was recently shown by one of us (Rabinowitch, '38 b). That much of the improvement subjectively is, however, also due to improvement of the protein metabolism is suggested from comparison of diets 5 and 6. It will be noted that there was a further retention of nitrogen without the between-meal feedings by increasing the intake of protein from about 70 to about 100 gm. a day.

Comparison of diets 6 and 7 shows the value of between-meal feedings even with liberal amounts of protein whenever it is necessary to reduce the caloric content of the diet. It will be noted that though the intake of protein was adequate and the same with both diets (both contained approximately 100 gm. of protein, two-thirds of which was of animal origin) the between-meal feedings of 10 gm. of carbohydrate resulted in a still further retention of nitrogen. Diet 8 shows that a still better result was obtained when the between-meal feedings consisted of 20 gm. of carbohydrate.[1]

Three examples of the combined effects of a) the high level of protein intake, b) the high proportion of animal protein, c) the liberal amount of carbohydrate, d) the small amount of fat, and e) the between-meal feedings are shown in table 2. Children were purposely selected to demonstrate the force of this combination, because of the importance of protein storage for growth and development in general. In the past, as diets 1 to 3 clearly show, nitrogen equilibrium was maintained with very great difficulty in diabetics, though the urines were kept free of sugar and the blood sugars were kept perfectly normal. With hyperglycaemia and glycosuria, storage of nitrogen, judging from the literature and from our own experiences, was a rare phenomenon. The data in table 2

[1] The 20-gm. meal was not accidental; it was based upon the observation by Haggard and Greenberg that 20 gm. of carbohydrate resulted in the maximum increase of net muscular efficiency (personal communication).

are, therefore, significant in that, in spite of the average urinary excretions of 23.6 and 41.0 gm. of sugar per day, the average intake of nitrogen exceeded the average output in two of these cases (6051/32 and 3022/36) by 0.06 and 0.17

TABLE 2

Showing retention of nitrogen in spite of post-prandial glycosuria[1] *in severe juvenile diabetics treated with the high carbohydrate-low calorie diet*

REMARKS	6051/32	3022/36	5193/38	
Age (yrs.)	6	12	7	
Diet				
Carbohydrate	230	218	210	
Fat	45	42	45	
Protein	72	99	70	
Total available glucose	276	280	255	
Calories	1613	1646	1525	
Average weight (kg.)	20.0	23.1	20.2	
Protein				
a) Total: (gm./kg.)	3.6	4.3	3.5	
b) Animal: Gm.	48.6	72.0	42.0	
Per cent total	67.5	72.7	60.0	
Gm./kg.	2.4	3.1	2.1	
Days of observation	14	12	12[2]	14[3]
Insulin (units/24 hours)	20	45	30.8	35
Urine sugar (gm./24 hours)	23.6	41.0	12.4	0
Nitrogen balance				
Intake (gm.)	11.5	15.8	11.2	11.2
Output (gm.)	10.3	11.8	6.5	7.7
Retention (gm.)	1.2	4.0	4.7	3.5
Retention (gm./kg.)	0.06	0.17	0.23	0.17
Intake/output × 100	1.12	1.34	1.72	1.45

[1] Urine free of sugar and blood sugar normal in fasting state.
[2] Period of glycosuria.
[3] Period of no glycosuria.

gm. respectively per kilogram of body weight. That these children were not mild diabetics is clearly shown by comparison of their ages with their insulin requirements.

With diets of total available glucose values of 276 and 280 gm., average excretions of 23.6 and 41.0 gm. of sugar are not very great losses and, as was shown in another report,[2] differ-

[2] Rabinowitch, I. M. The significance of post-prandial glycosuria. Ann. Int. Med. In press.

ing from the experiences with low-carbohydrate-high fat diets of the past, post-prandial glycosuria is not harmful with the high carbohydrate-low fat diet, providing the blood sugar is normal in the fasting state. Glycosuria is, however, harmful when there is hyperglycaemia in the fasting state. The data of the third child (5193/38) are, therefore, instructive in that storage of protein occurred in spite of the poor control of the diabetes, as shown by the degree of hyperglycaemia in the fasting state. It will be noted that when the data were divided into two periods, namely, a) when the urine was free of sugar and b) when the urine contained sugar, there was no greater—in fact there was less—retention of nitrogen during the sugar-free period than when the urine contained sugar. The maximum, minimum and average blood sugars in the fasting state, in the corresponding periods, were 0.277, 0.217, 0.243 and 0.250, 0.175 and 0.204% respectively. In view of the experiences with the old high fat-low carbohydrate-low protein diets, the explanation of the storage of the protein appears to be enhancement of the protein-sparing action of the carbohydrates by the between-meal feedings, combined with the influence of the high carbohydrate-low fat intake and the liberal supply of protein, quantitatively and qualitatively.

The above nitrogen balances fit in with our experiences with skeletal growth in juvenile diabetics. Two examples are cited in figures 1 and 2. Figure 1 is that of a girl age 12 years, 8 months (1528/35) who, prior to her admission to this clinic, had been treated for the diabetes by drastic reduction of food. According to the description of her diet she received approximately 1.5 gm. of protein per kilogram of body weight and the protein of animal origin accounted for approximately 1.1 gm. per kilogram. It will be noted that she was markedly underheight, according to her age (M. S. Rose, '38)—the actual and expected heights were 48.0 and 55.5 inches respectively. Shortly after the carbohydrate content of the diet was increased and the total protein was increased to 3.0 gm., and the animal protein to 2.2 gm., per kilogram, she

began to grow rapidly and, at the end of about 3 years, the actual height was within about 2 inches of the expected (she gained 9½ inches in 35 months) whereas, prior to the change of diet it was, as stated approximately, 7½ inches below that expected for her age.

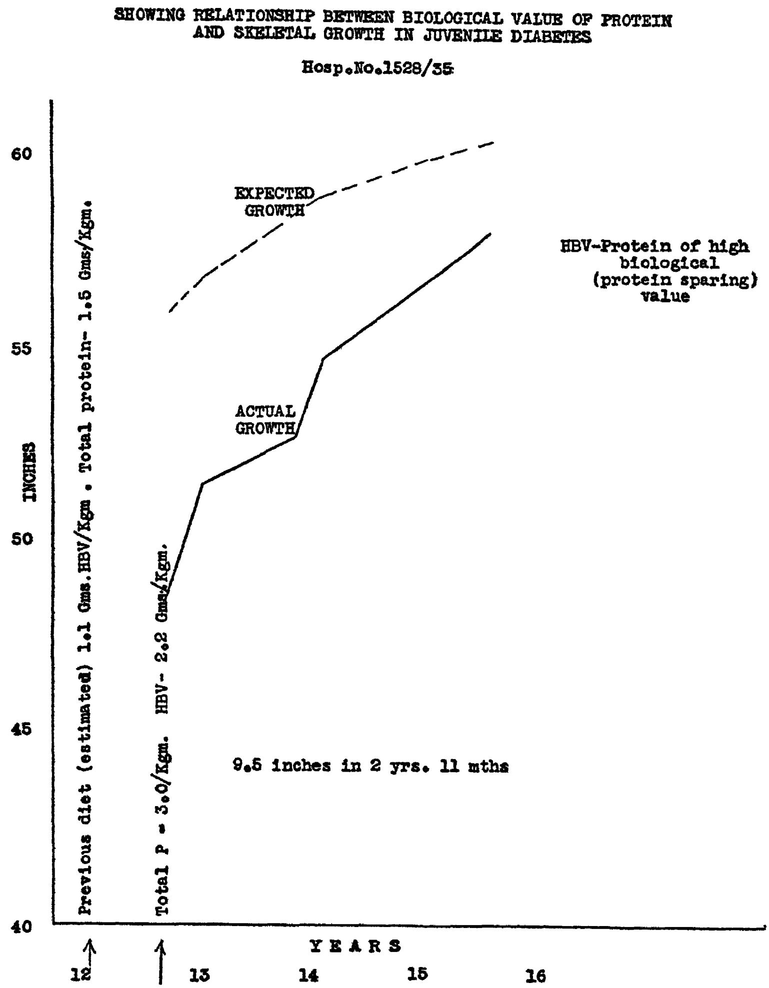

Figure 1

Figure 2 is that of boy (70/28) who, at the age of 7 years, was normal with respect to height. The total protein content of the diet appeared to be adequate, but it will be noted that, until the age of about 9½ years, he failed to grow at the normally-expected rate and, from 9½ to 12½ years, the height

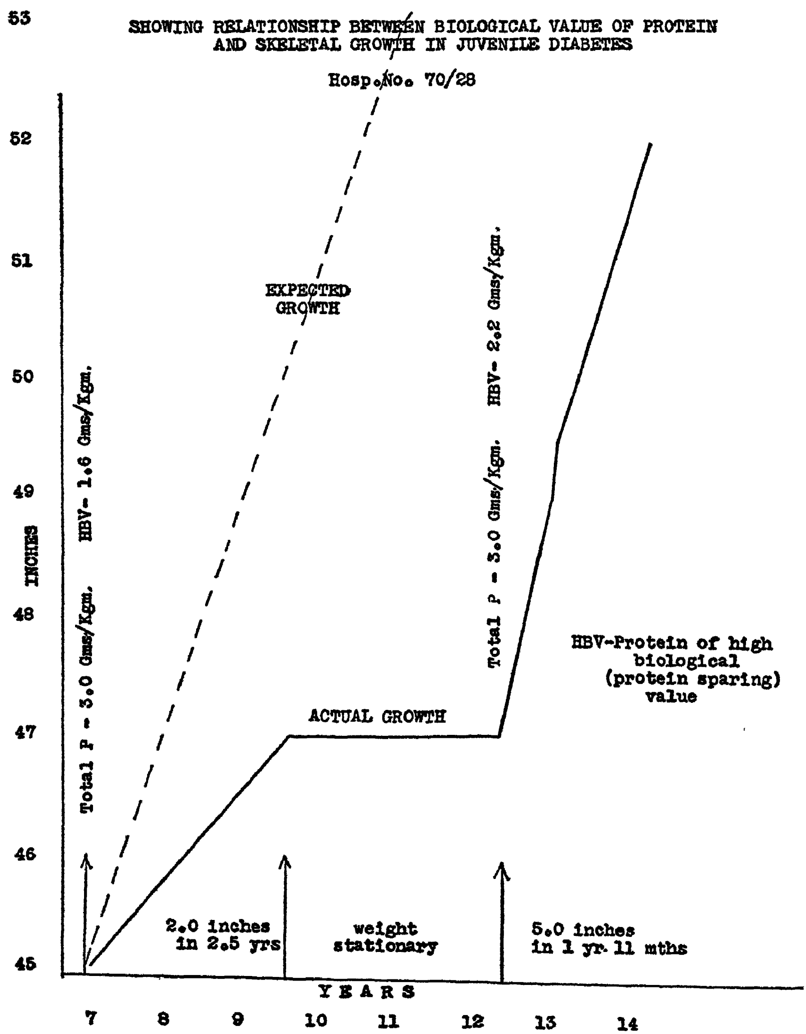

Figure 2

remained stationary. The animal protein was then increased from 1.6 to 2.2 gm. per kilogram of body weight. Thereafter, he began to grow at a very rapid rate and gained 5 inches during the next 23 months.

In view of these experiences with skeletal growth, it is appropriate to emphasize the importance of the above-mentioned variables which should be considered in the construction of low-calorie diets, particularly because of the increasing literature with respect to the therapeutic use of growth hormones. There appears to be no doubt that growth hormone therapy may at times be effective in the diabetic (Beck and Suter, '38) as well as in the non-diabetic (Lawrence and Harrison, '38; Molitch and Poliakoff, '38). According to our experience with juvenile diabetes, however, nutritional dwarfism is very much more common than true pituitary dwarfism and is correctable by diet alone. In a statistical study of skeletal growth among our juvenile diabetics 10 years ago (Rabinowitch and Bazin, '29) it was shown that though the children, in general, were overheight before they developed their diabetes and thus conformed to the generally-recognized pattern (White, '27) they ceased to grow rapidly soon after the institution of the treatment of the diabetes. It was then suggested that the control of the diabetes also tends to control the excess activity of the growth hormone. Since then, however, with more knowledge of the protein metabolism in such cases, there is much more reason to believe that the reduced rate of growth then noted was not due to control of the diabetes, but to the diets which, because of their deficiencies, did not permit storage of adequate amounts of protein.

SUMMARY

A study was made of the nitrogen metabolism of diabetics treated with low calorie diets but with different amounts of carbohydrate and fat and different amounts of vegetable and animal proteins.

The findings fit in with previous observations that carbohydrates tend to enhance, whereas fats tend to interfere with

storage of protein in the body; and that the latter is not only also influenced by the total amount of protein in the diet, but also by the relative amount of protein of high biological (protein-sparing) value.

The observation made with diets of normal caloric content that time is a factor which influences the protein-sparing action of carbohydrates is confirmed with diets of low caloric contents.

The many practical applications of these newly-established facts of nutrition are suggested from the many people who for different reasons in health or disease must reduce the caloric contents of their diets. Experiences with skeletal growth in diabetic children are cited as examples.

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A NEW LOW FLUORINE DIET AND ITS EFFECT UPON THE RAT [1, 2]

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Attempts have been made in several laboratories to produce fluorine-free diets. Sharpless and McCollum ('33) prepared a highly purified diet which they state "was very low in fluorine but not quite free." Marcovitch, Shuey and Stanley ('37) used rice and milk to obtain a ration which contained about 0.6 part of fluorine per million. Fellenberg ('37) and Tamman (1888) have reported traces of fluorine in milk. Repeated analyses of milk made in this laboratory have shown that it contains between 0.1 and 0.2 parts of fluorine per million (Phillips, Hart and Bohstedt, '34). Since milk can be converted to an adequate diet by mineralization it would seem that mineralized milk would offer an excellent means of studying the effects of a low fluorine diet upon the rat.

EXPERIMENTAL

These experiments were designed to study the effect of this low fluorine diet upon the skeletal fluorine, growth and reproduction in the rat. Fresh milk was mineralized with 1 mg. of iron ($Fe_3(SO_4)_2$), 0.1 mg. of copper ($CuSO_4$) and 0.1 mg. of manganese ($MnSO_4$) per 100 cc. Young rats weighing

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[3] Moorman Manufacturing Company fellow.

40 gm. each were divided, one male and three females per lot, as follows:

Lot	
I	Basal ration—mineralized milk
II	Basal + 0.1 p.p.m. fluorine as NaF.
III	Basal + 1.0 p.p.m. fluorine as NaF.
IV	Basal + 10.0 p.p.m. fluorine as NaF.
V	Basal + 20.0 p.p.m. fluorine as NaF.
VI	Basal + 20.0 p.p.m. aluminum as $AlCl_3$.
VII	Basal + 20.0 aluminum as $AlCl_3$ + 20.0 p.p.m. fluorine as NaF.
VIII	Basal + 20.0 p.p.m. fluorine as NaF + 3 drops of percomorph oil per rat per week.

In this experiment an attempt was made to determine if aluminum or excess vitamin A would reduce the effects of the higher levels of fluorine. It was planned that the animals on experiment would be raised to maturity and allowed to reproduce. The animals were kept on raised wire screens except when pregnant females were ready to litter. In the latter case the females were put on shavings. All attempts to raise litters on screen floors failed. At birth the litters were reduced to four pups. The remaining pups were taken immediately for fluorine analyses. At 21 days one young rat from each lot was likewise taken for analyses to determine if a large increase in fluorine concentration occurred during the suckling period. The remaining young were used for the production of a second generation. A third and fourth generation were likewise obtained. The adult rats were removed when the succeeding generation rats were produced. Some remained on experiment 4 and some 7 months. In these cases the tibia and femurs were analyzed to determine the skeletal fluorine content.

The fluorine analyses were made by the modified method of Willard and Winter as previously described (Evans and Phillips, '38).

RESULTS

Mineralized milk proved to be a good low fluorine ration. On this diet the rat remained healthy and vigorous through

five generations. Food consumption records showed that only 0.05 to 0.06 mg. of fluorine per kilogram of body weight were ingested per day. It is plainly evident that in this species, which can on occasion withstand relatively large quantities of fluorine, very little fluorine is necessary.

In these experiments the growth records of the young were alike. There was no retardation of growth when 20 parts of fluorine per million were fed. Neither did the added aluminum or percomorph oil affect growth. On the basal ration plus 20 p.p.m. of fluorine the incisor teeth were definitely bleached and elongated. Food consumption indicates that this occurred on an intake of 5 mg. of fluorine per kilogram of body weight per day. The marginal level where bleaching was first noticed was found to be 10 p.p.m. At this level the rats ingested 2.5 to 3.0 mg. of fluorine per kilogram of body weight per day. Above 3.0 mg. of fluorine per kilogram of body weight per day bleaching always occurred. Below this level the teeth were normal in appearance. No beneficial results attended the addition of 0.1 or 1.0 part of fluorine per million to the milk. Neither aluminum at the level fed here nor the percomorph oil prevented bleaching of the incisors.

Reproduction difficulties were observed during mid- and late winter. This explains why the second generation required 7 months to produce the third generation. In general the levels of fluorine administered had no beneficial or adverse effect upon reproduction. Failure occurred in lot V but previous experience had shown us that rats on this ration would reproduce for three consecutive generations. Lots VI and VII also failed to reproduce beyond the first generation.

Table 1 shows the average fluorine content of the young rats by generations. It is evident that the newborn in lot I on the basal ration alone contained appreciable fluorine. This residual fluorine was not greatly affected until the added fluorine reached 10 or more parts per million, and then there seemed to be a rather distinct increase in the first generation except in one case (lot VIII). There was no significant difference between the fluorine content of the young of the first

and third generations on the respective levels of fluorine fed. There seemed to be no cumulative effect carried over from generation to generation and this applies to both the lowest and the highest levels administered. Unborn fetuses gave similar fluorine contents to the day-old young. This indicates that the young at birth were not greatly contaminated with fluorine.

It has been estimated from our analytical data that 33% of the basal ration fluorine ingested during the gestation period was deposited in the fetus. The actual dry matter represented in the fetuses was only 5.1% of the total solids

TABLE 1

The average fluorine content of young rats (fluorine in p.p.m.)

	LOT I BASAL	LOT II BASAL + 0.1 ppm F	LOT III BASAL + 1.0 ppm F	LOT IV BASAL + 10 ppm F	LOT V BASAL + 20 ppm F	LOT VI BASAL + 20 ppm Al	LOT VII BASAL + 20 ppm F + 20 ppm Al	LOT VIII BASAL + 20 ppm F + perco-morph oil
At birth								
F1	12.0	8.5	15.6	19.2	19.2	7.0	23.6	6.7
F2	..	8.8	..	24.5	..	..	..	47.7
F3	10.6	10.3	11.3	21.4	..	..	..	19.0
At 21 days								
F1	5.7	8.4	7.8	25.0	7.2	6.0	5.9	5.2
F2	4.1	4.0	..	24.6	..	..	..	24.6
F3	3.5	5.3	6.2	22.0	..	..	..	46.7

ingested. Thus the residual fluorine in the fetus seems to be larger than one would expect from the fluorine content of the ration. This suggests a preferential storage of the milk fluorine in the fetal tissues. This suggestion is further supported by the fact that females which reproduced contained smaller amounts of skeletal fluorine than their respective lot mates. These data show a definite placental transfer of fluorine.

It is also seen from table 1 that there was comparatively little storage of fluorine during the suckling period. The fluorine increase from birth to 3 weeks was slightly more than doubled. The dry matter of the rat at birth averaged 1.0 gm.

and contained 10 µg. of fluorine. At 21 days the dry matter averaged 5.0 gm. and the fluorine content 22.5 µg. Thus the proportion of fluorine stored in the rat in comparison to the increase in body weight was considerably less during the suckling period. There was no increase in fluorine storage during the suckling period when the mother received as much as 20 p.p.m. of added fluorine.

Table 2 shows the relative skeletal storage of fluorine with respect to the fluorine ingested by the various lots. On the basal ration alone the average skeletal fluorine was 8 to 16 p.p.m. irrespective of the length of time on experiment. The addition of an equivalent amount of fluorine as NaF to that

TABLE 2

The average skeletal fluorine content of the adult rats (fluorine in p.p.m.)

GENERATION	AGE MONTHS	LOT I BASAL	LOT II BASAL + 0.1 ppm F	LOT III BASAL + 1.0 ppm F	LOT IV BASAL + 10 ppm F	LOT V BASAL + 20 ppm F	LOT VI BASAL + 20 ppm Al	LOT VII BASAL + 20 ppm F + 20 ppm Al	LOT VIII BASAL + 20 ppm F + percomorph oil
F	4	16	72	297	1639	2562	71	2339	2335
F1	7	16	44	355	2781	4522	42	4067	4527
F2	4	16	54	265	1729	..	..	..	3065
F2	7	8	54	341	2695	..	..	..	4063
F3	4	10	71	265	1573	..	..	..	2992
F intake mg./kilo/day		0.05–0.06	0.075–0.082	0.30–0.42	2.5–3.2	5.05	0.05	5.05	4.9–6.3

already present in the basal ration resulted in more than a threefold increase in the skeletal storage. When the added fluorine was increased to 1.0 p.p.m., however, the portion stored was distinctly not linear. It appears that the fluorine naturally occurring in the milk is less readily available for storage in the skeleton. Again there seem to have been no significant changes in the skeletal fluorine from generation to generation regardless of the dietary level fed. Certainly the third and fourth generation rats on this low fluorine diet had bones similar in fluorine content and gross appearance to those of the first. These observations again point to the fact

that this ration was adequate in its fluorine content for the rat.

When 1.0 or more parts of fluorine per million were fed, there was a greater storage of fluorine in the skeleton during a 7-month period than there was during a 4-month period. Twenty parts of aluminum per million gave a slight protective action against the storage of fluorine in the skeleton and this protective action was more noticeable with the increase in the length of the experimental period. This level of aluminum did not protect against bleaching of the teeth. Higher levels of aluminum (100 p.p.m.) on this diet do protect over a 2-month period. Aluminum added to the basal diet did not prevent the fluorine present in the milk from being stored in the skeleton even though the ratio of aluminum to fluorine reached the figure of 40:1. Vitamin A in these experiments was not efficacious in reducing the fluorine deposited in the skeleton.

DISCUSSION

The data presented here indicate that excellent results have been obtained using milk as a low fluorine diet. On the basis of dry matter this ration contained 1.6 p.p.m. The skeletal fluorine averaged 13 p.p.m. with a range from 1 to 31 p.p.m. Sharpless and McCollum ('33) obtained skeletal fluorine contents varying from 6 to 28 p.p.m. with an average of 21 p.p.m. on their diet. Marcovitch, Shuey and Stanley ('37) obtained an average of 36 p.p.m. in the skeletons of their rats which were on experiment for a shorter length of time than those in these experiments.

These results show that fluorine is transferred through the placenta. This agrees with our results obtained with cattle (Evans, Phillips and Hart, '38). Whether these results indicate that fluorine is necessary for the developing young is not known. Previous work (Evans, Phillips and Hart, '38) has shown that extraordinary amounts of fluorine are present in embryonic cartilage ash and the organic matrix of bones.

The physical appearance of the bones from the animals fed only the basal ration appeared to be normal in every respect.

The bones were strong, smooth, evenly and well calcified. It appears that the low fluorine found in milk does not produce an inferior bone.

SUMMARY AND CONCLUSIONS

Mineralized milk proved to be a good low fluorine diet through five generations of rats. Summer milk properly mineralized supported reproduction while winter milk was inferior in this respect.

Fluorine is not necessary for the rat in amounts larger than the 0.1 to 0.2 p.p.m. found in milk. Thus 50 μg. of fluorine per kilogram of body weight per day meets all of the requirements of the rat for growth, reproduction and general well being. Additional fluorine from 0.1 to 20.0 p.p.m. caused no measureable improvement in the rat.

The borderline zone for bleaching of the teeth occurred at a level of 10 p.p.m. Bleaching was not prevented by aluminum or the extra vitamin A furnished by percomorph oil used in these experiments.

There was a placental transfer of fluorine even on the low level of fluorine ingested. This was not increased until the level of fluorine reached 10 p.p.m. Thereafter there was a definite increase in fluorine passing through the placenta. Mammary secretion of fluorine, on the other hand, was not affected by as much as 20 p.p.m.

The low level of fluorine furnished by the basal milk diet did not deplete the fluorine stores of the rat through five generations, nor did it increase the demand for fluorine. There was no cumulative fluorine effect carried over from generation to generation on either the lowest or the highest level.

The fluorine normally present in milk appeared to have been less readily metabolized for storage in the skeleton than that added as sodium fluoride.

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INDIAN AND ESKIMO METABOLISMS

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During the summer of 1937 members of the Cleveland Clinic Expedition to northeastern Canada and the subarctic regions of Churchill Bay and Chesterfield Inlet made a series of metabolism studies on thirteen Chippewa Indians living on a reservation near Churchill and on sixty-three Eskimos living in the vicinity of Chesterfield Inlet. Six of the Indians were males and seven were females; of the Eskimos, thirty were males and thirty-three were females. A number of the tests included here were made by Dr. Thomas Melling, Medical Health Officer for the District of Keewatin, Canada, who has his headquarters at Chesterfield. After having been instructed in the use of our Jones metabolism apparatus he carried on many of the tests on the Eskimos.

In each instance a short preliminary test was made with the individual undergoing the test presumably under basal conditions. Following this, the next test was recorded and it is this record in every case which is represented in our metabolism data. The individuals tested were instructed to eat no food following their evening meal on the day prior to the test. From the appearance of the Indians' tents on the morning of the test plus their apparent willingness to cooperate with us induced in part, perhaps, by the promise of small presents, we believe that they obeyed this injunction against taking food. Doctor Melling followed this same general procedure with the Eskimo subjects and found no difficulty in securing their cooperation. Besides this, he is well known to them and enjoys their confidence. Since the matter of having

meals at a set time is of no great moment to either Indian or Eskimo, this request to forego breakfast combined with an admonition to rest prior to the test, involved no hardship for the subjects of the test.

BASAL METABOLISM TESTS ON CHIPPEWA INDIANS

The tests upon the Chippewa Indians were carried out on warm, sunshiny days in early August. The tests were made upon a wide bench in the open, but sheltered from the wind. Before the test the individuals reclined in their tents and remained so until called for the tests.

A word might be said with reference to the habits of the Chippewa Indian in this region. He lives in tents the year around, he does a little hunting and fishing and counts on the Canadian Government for support in the event that his own efforts fail to provide him with sufficient food and clothing to maintain himself. Apparently he makes less effort to fend against the inhospitable climate than does the Eskimo, and he does not show the same adaption to the rigors of northern life as does the Eskimo. His food consists of fish and game, eked out with 'store' food where he can procure it.

Table 1 sets forth the data as secured on the Indian males and females. Included in these data are age, weight, height, as well as blood pressure, temperature, pulse rate and metabolism percentage based upon the Mayo normal standard records as established for white individuals of corresponding age, sex and body size.

METABOLISM

Disregarding the values over +40 in the male as well as in the females, the average metabolic values are +18% for the male Chippewa Indian and +18.50% for the female Chippewa Indians. These values we believe to be approximate basal values for this group.

PULSE AND BLOOD PRESSURE

We were surprised at the slow pulse rate in the Indian. This ran significantly lower than did the pulse rate of the

Eskimo. The average pulse rate for the Indian males was 53.3 against 70 for the male Eskimo and 63 for thirty-five Maya-Quiché Indian males whose records were secured in Guatemala. In the female Indian the average pulse rate was 65 as compared with 78.8 for the Eskimo female and 82 for five

TABLE 1

Chippewa Indians

Males

NUMBER	AGE	HEIGHT	WEIGHT	TEMPERATURE	BLOOD PRESSURE		PULSE	O_2 CONSUMED PER MINUTE	DEVIATION FROM MAYO NORMAL STANDARD
	Years	*cm.*	*kg.*	*F.*	*Sys.*	*Dias.*		*cc.*	*%*
1	21	163.8	61.2	96.8	112	78	55	258	12[1]
2	40	176.9	70.3	97.0	102	75	42	...	..
3	33	170.8	60.3	97.0	128	85	48	250	9
4	28	166.4	60.3	96.5	118	78	56	266	16
5	24	166.4	63.0	96.3	134	80	62	320	35
6	30	168.9	65.8	95.8	126	85	58	333	44
Av.	29	168.8	63.4	96.9	120	80	53.3	273	+18.0 without no. 6
				Female Indians					
7	15	157.5	48.5	98.0	122	74	52	216	5
8	17	158.7	54.9	97.8	116	72	62	333	64
9	50	166.4	68.5	97.2	120	70	72	253	23
10	33	160.0	60.3	97.4	145	86	60	266	39
11	50	151.8	61.7	98.4	114	72	78	210	8
12	30	152.3	58.9	97.0	128	78	54	235	22
13	33	157.4	66.2	97.4	126	76	78	235	14
Av.	33	157.7	59.8	97.6	124	75	65	236	+18.5 without no. 8

[1] All values in final column are 'plus' unless otherwise indicated.

Maya-Quiché Indian females. The pulse of these northern Indians is characterized by the fullness, and regularity of the beat.

The blood pressure of the Chippewa Indians averaged 120/80 for the males and 124/75 in the females as against 119/75

for the Eskimo males and 112/72 in the Eskimo female compared with 111/77 for thirty Maya-Quiché Indian males and 111/75 for five Maya-Quiché females in a series of tests made in Guatemala, C. A.

Considering the nomadic habits of these northern Chippewa Indians, their extreme endurance, which in many instances rivals or exceeds that of the Eskimo in the sense that they face the extreme rigors of the climate and yet fail to provide the warm shelters for themselves as do the Eskimos, and by their failure to clothe themselves as warmly as do the Eskimos either in summer or in winter, the relatively high metabolic rate might be explicable on these grounds. Considering the effect of environmental temperature upon the basal metabolic rate, any continued exposure to the prevailing temperatures at the latitude of Churchill, which marks the northern limit of the Indian, might be expected to raise this rate significantly. On the other hand, the Maya-Quiché Indian of Central America according to our own findings and those of Steggerda, Williams, Shattuck, Benedict, the Navajo Indian of Arizona according to Salsbury, all tend to show a basal metabolic rate significantly higher than the standard estimates for whites of similar ages and weights. It is evident that this higher basal rate is not a Mongolid characteristic since the studies of MacLeod, Crofts and Benedict, Knipping, Takahira, all show the southern Mongolid types (the Sinid and the Palaeomongolid) run a lower basal metabolic rate than do the Indians or the Eskimos measured on this continent which presumably are an offshoot of the North mongolid or Tungid type of the human race.

BASAL METABOLISM TESTS ON ESKIMOS

The Eskimos represented in this study live almost entirely on a native diet according to Doctor Melling. Their food consists of caribou, seal, walrus, fish and birds. They still live in caribou skin or canvas tents in summer and snow huts in the winter.

Table 2 represents the data secured from thirty male Eskimos between the ages of 15 and 85 years with an average age of 36.8 years. The average nude weight of these Eskimos was 64.9 kg., or approximately the same as prevails for male whites in the United States. The average male height of 5 feet 5 inches is somewhat under the white average.

TABLE 2

Eskimo males

NUMBER	AGE	HEIGHT	WEIGHT	TEMPERATURE	BLOOD PRESSURE		PULSE		O_2 CONSUMED PER MINUTE	DEVIATION FROM MAYO NORMAL STANDARD
	Years	*cm.*	*kg.*	*F.*	*Sys.*	*Dias.*	*Bef.*	*Aft.*	*cc.*	*%*
14	47	165.1	72.5	97.4	118	86	54	66	250	8[1]
15	85	161.3	57.6	97.0	92	70	67	84	206	8
16	40	170.2	72.5	97.0	138	80	72	60	308	27
17	18	155.0	75.2	96.4	122	78	66	84	286	24
18	22	170.2	63.9	98.0	148	80	72	84	286	17
19	28	155.0	58.1	98.3	116	72	64	62	235	9
20	30	160.0	67.1	96.6	124	70	64	70	242	5
21	37	157.4	66.7	96.3	162	90	62	68	286	29
22	40	157.4	58.1	96.3	108	64	60	60	235	13
23	70	172.1	73.5	96.3	112	70	68	64	250	11
24	50	158.1	62.1	96.1	122	70	80	78	285	35
25	65	167.7	64.4	96.0	120	60	78	72	235	13
26	15	153.7	48.5	96.3	120	65	72	80	242	4
27	30	158.8	50.8	97.4	112	78	60	56	266	18
28	64	165.1	75.7	97.0	120	84	66	60	333	42
29	50	165.1	77.1	96.0	114	78	60	68	258	9
30	55	161.3	61.7	96.2	138	98	80	76	258	21
31	22	157.7	58.9	98.1	102	68	72	72	242	8
32	24	163.8	63.0	98.0	104	68	80	80	273	16
33	45	175.2	78.0	97.3	116	78	72	56	286	14
34	55	165.1	72.1	96.4	102	68	60	56	250	12
35	25	159.4	64.4	97.6	138	86	60	60	273	18
36	35	171.5	72.1	96.0	118	84	76	60	266	7
37	28	166.3	75.3	97.1	126	88	76	76	286	14
38	40	158.1	58.5	97.1	128	78	72	72	250	21
39	18	157.5	46.3	97.3	96	78	80	80	242	18
40	18	160.7	54.9	96.8	116	62	64	64	250	8
41	38	160.0	71.2	96.6	108	60	56	64	286	23
42	38	171.5	78.5	96.0	122	78	60	60	363	42
43	18	160.0	60.3	96.0	102	64	72	72	235	—3
Av.	38.3	162.7	65.3	96.8	118.8	75.1	61.9	68.8	265.3	+14.5 without +42 values; +16.4 including all values

[1] All values in final column are 'plus' unless otherwise indicated.

TABLE 3

Eskimo females

NUMBER	AGE	HEIGHT	WEIGHT	TEMPERATURE	BLOOD PRESSURE		PULSE		O_2 CONSUMED PER MINUTE	DEVIATION FROM MAYO NORMAL STANDARD
	Years	*cm.*	*kg.*	*F.*	*Sys.*	*Dias.*	*Bef.*	*Aft.*	*cc.*	*%*
44	47	152.4	58.9	97.0	124	80	66	66	266	42[1]
45	18	148.5	51.3	98.0	122	78	90	84	235	29
46	16	147.9	53.1	98.0	126	80	72	90	286	45
47	21	151.1	55.8	98.1	128	78	72	78	258	38
48	42	153.7	56.6	98.3	98	68	96	84	258	43
49	56	154.9	57.2	97.2	124	72	84	66	222	26
50	17	144.7	45.8	97.2	104	68	60	72	216	23
51	16	138.4	44.4	98.5	114	72	58	74	308	76
52	38	149.8	55.3	97.5	125	78	70	80	205	12
53	50	152.4	61.0	96.1	120	68	76	68	250	35
54	45	150.4	55.3	97.3	108	70	80	76	195	8
55	45	158.1	54.0	95.5	118	78	80	76	205	11
56	16	154.9	52.2	97.3	108	68	92	96	222	10
57	35	153.0	54.0	96.1	136	80	84	92	258	40
58	18	161.3	46.7	97.0	102	70	88	84	222	19
59	22	156.2	66.7	97.0	124	88	84	84	258	25
60	55	151.1	53.1	97.0	130	86	80	84	222	31
61	45	140.9	51.3	98.1	138	80	76	84	235	40
62	40	151.1	58.9	98.1	108	74	64	76	286	51
63	20	148.5	53.1	97.0	108	64	76	80	222	23
64	18	158.1	59.4	97.0	108	68	84	80	206	0
65	45	143.5	50.3	97.0	112	66	64	76	266	58
66	18	157.3	67.0	97.4	116	70	76	88	235	9
67	27	146.7	55.8	96.2	110	72	80	76	222	23
68	35	152.4	61.2	98.3	110	70	100	104	242	23
69	45	160.6	57.6	98.0	88	68	68	68	222	20
70	22	163.2	58.5	96.4	90	70	76	80	348	70
71	16	151.1	48.5	97.8	96	68	92	92	216	13
72	19	147.0	59.9	97.0	102	58	64	52	222	15
73	30	151.1	49.0	96.3	98	64	64	84	222	26
74	40	152.4	54.1	98.6	104	68	92	96	235	29
75	20	148.5	55.3	98.1	118	64	96	116	235	27
76	40	147.9	56.2	97.2	94	60	108	100	242	31
Av.	31.4	151.5	55.1	97.3	112.1	71.7	78.8	82.4	240.5	+21.12 without 40 or over values; +29.4 including all values

[1] All values in final column are 'plus' unless otherwise indicated.

The average basal metabolic rate for these male Eskimos excluding values over +40 was +14.5. Including all values the average was +16.4.

Table 3 shows the data secured from thirty-three female Eskimos. The age range in this group was from 16 to 65 years with an average age of 32.4 years. The average nude weight of the group was 55 kg. or just about the average for American female whites.

The average metabolic rate recorded for the entire group of female Eskimos was +29.4. Omitting all values of +40 or over the average values of the remaining 24 individuals was +21.12.

TABLE 4

GROUP MALE AND FEMALE	MEAN METABOLISM PER CENT + B. M. R.	STANDARD DEVIATION	COEFFICIENT OF VARIATION PER CENT
Male and female Indians	18.25±3.46	10.90±2.55	58.8±10.31
Male Eskimos	14.50±1.62	8.50±1.13	58.46± 7.81
Female Eskimos	21.12±1.76	8.65±1.25	38.41± 5.54

PULSE AND BLOOD PRESSURE

The pulse rate of the male Eskimo, 63 to 69 as well as that of the female at 79 to 83 falls perhaps slightly under the pulse values of white males and females and is markedly higher than that of the Chippewa Indians.

The average blood pressure for the males was 119 systolic, 75 diastolic and for the females it ran 112 systolic and 71 diastolic. The average height of 5 feet ½ inch appears to be considerably lower than the white female average.

SUMMARY

Metabolism tests were made on six male and seven female Chippewa Indians. The average metabolic values recorded for the males was +18.0% for the females it was +18.5%. Pulse rate and blood pressure both appear to be lower than they are for the American white population or the Eskimo, or Mayan Indian of Central America.

The metabolism tests run on thirty male Eskimos from the region around Chesterfield Inlet, Canada, gave an average reading of +16.4% for the males and +29.0% for thirty-three females when the values above +40 are included; without the values above +40% the averages for the males are +14.5% and for the females they are +21.12%. The blood pressure for both the males and the females is lower than that of whites of corresponding age, the pulse rate corresponds rather closely to that of white individuals.

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A STUDY OF THE METABOLISM OF THE MAYA QUICHÉ INDIAN

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During a recent expedition to Guatemala, Central America, 164 basal metabolism estimations were made on thirty-five male Maya Quiché Indians. Thirty of the subjects were soldiers with from 6 months to a year of army service. These men were stationed at Totonicipan at an altitude of 8100 feet. Five were coffee plantation laborers stationed on the Pacific slope at an altitude of 800 feet. In addition, eight tests were made upon one white male and one white female at 8100 feet and on one white male living at 800 feet altitude. All the tests were run in December, 1938, and January, 1939. The study on the soldiers was made possible through the courtesy and the assistance of the governor of Totonicipan Province, Colonel Carlos Cipriani.

To test the possible stimulatory effect of chili, twenty tests were run on ten soldiers 3 hours after they had ingested measured amounts of chili. As a check on the chili tests six subjects were tested 3 hours after eating from 1 to 10 gm. of tortillas (corn cakes). From four to eight tests were made upon each subject in the course of a week. The usual data necessary for making metabolism determinations were taken. The soldiers were tested in their barracks and after the initial test took little interest in the proceedings commonly falling asleep while waiting their turn to be tested. Following the evening meal on the night before the test, they remained in their bunks until the conclusion of the tests. During the tests

TABLE 1

Maya-Quiché metabolism data

NUMBER	AGE	HEIGHT	WEIGHT	TEMPERATURE	BLOOD PRESSURE		PULSE		NUMBER OF TESTS	O_2 CONSUMED PER MINUTE	DEVIATION FROM MAYO NORMAL STANDARD	PERCENTAGE CHANGE OVER SUBJECT'S OWN BASAL RATE AFTER CHILI FEEDING	PERCENTAGE CHANGE OVER SUBJECT'S OWN BASAL RATE AFTER TORTILLA FEEDING	DOSAGE OF CHILI OR TORTILLA
	years	*cm.*	*kg.*	*F.*	*Sys.*	*Dias.*	*Bef.*	*Aft.*		*cc.*	%	%	%	
1	22	154	54.0	98.6	111	79	61	64	7	242	+13	+10.8		1 gm. dry chili
2	18	154	50.1	97.5	108	77	58	60	6	232	+7			
3	26	161	62.5	98.0	105	79	80	86	5	258	+12			
4	22	154	55.2	97.5	128	85	56	48	7	222	+5		−2.86	10 gm. tortilla
5	19	159	57.1	97.6	105	81	64	56	7	240	+5	+11.5		1 gm. dry chili
6	22	157	54.9	97.8	108	77	64	48	6	222	+6			
7	19	162	61.5	97.2	122	85	56	54	7	244	+6	+13.3		11 gm. liquid chili = 3 gm. dry
8	22	158	56.1	97.0	108	90	58	52	8	243	+14		0.0	10 gm. tortilla
9	24	172	69.5	98.3	108	74	60	64	7	251	+2	+12.7		12.18 gm. liquid = 3 gm. dry
10	23	165	57.3	97.2	108	75	66	66	8	243	+7	+14.0		12.53 gm. liquid = 3 gm. dry
11	27	168	64.0	97.0	112	75	60	54	6	260	+10	+11.0		11.20 gm. liquid and corn = 3 gm. dry
12	21	166	69.0	98.0	100	73	66	60	4	280	+13			
13	22	152	53.9	98.6	118	85	90	96	4	243	+14			
14	19	157	62.0	98.4	128	77	62	62	4	235	0			
15	24	155	54.9	97.5	115	85	54	54	4	222	+6			
16	22	159	57.1	97.6	112	79	54	54	6	219	−1		0.0	10 gm. tortilla
17	24	155	55.2	97.2	108	65	48	52	6	236	+11		+10.8	10 gm. tortilla
18	20	161	57.1	97.6	110	70	68	64	4	234	+5			
19	20	160	64.0	97.8	115	71	66	68	6	254	+10	0.0		1 gm. dry chili
20	21	165	61.5	97.6	124	76	60	62	6	242	+3	10.6		1 gm. dry chili

21	24	168	65.0	98.0	108	75	60	66	4	237	0			
22	18	157	54.9	97.5	105	71	66	66	4	242	+11			
23	25	156	54.0	98.0	110	67	54	54	4	240	+13			
24	24	158	58.0	98.0	115	75	56	56	4	232	+7			
25	24	156	54.1	97.6	118	77	48	48	4	222	+5			
26	22	153	52.0	97.8	112	75	48	52	4	211	+1			
27	33	158	59.4	97.6	115	80	66	66	6	264	+22		—13.2	10 gm. tortilla
28	19	156	57.6	97.2	110	79	54	56	6	219	—1		+13.1	10 gm. tortilla
29	19	156	58.0	97.0	116	73	72	66	6	245	+8	+1.018		1 gm. dry chili
30	26	158	53.2	98.0	98	75	72	72	6	239	+16	—2.6		1 gm. dry chili
Average:														
	22.39	159.3	57.57	97.6	111	77	63	61	166		+8.20	+8.46	+1.30	
Coffee plantation laborers														
31M	20	149	42.0	97.4	105	75	60	57	6	212	+12			
33	23	167	52.1	97.5	108	79	66	63	6	243	+10			
35	19	167	48.0	97.5	95	70	64	61	6	229	+6			
37	22	165	50.1	97.4	114	73	60	58	6	200	—6			
39	24	159	57.1	97.4	108	68	62	63	6	228	+4			
Average:														
	21.6	161	49.8	97.4	104	73	62	60	30		+5.2			
White persons														
N.R(F.)	34	163	62.6	97.5	116	77	72	72	2	194	—6			
A.G(M.)	36	171	70.5	97.5	135	77	72	68	2	210	—13			
J.M(M.)	25	175	67.5	96.7	122	69	72	72	4	222	—12			
Average on two males:														
	30.5	173	69.0	97.1	128.5	73	72	70	8		—12.5			

[1] Unless indicated with a minus sign, all values are plus.

they were under constant observation. A Jones metabolism[1] unit provided with an oversized bellows and adapted for field work was used in this study.

DISCUSSION OF RESULTS

Tables 1 and 2 set forth the data secured and the mean metabolism rates respectively. The tests ranged from a −1 to +22% which represents the mean calculated percentage based upon the Mayo normal standard established for white

TABLE 2

Mean metabolism rates

LOCATION	NUMBER OF TESTS	SEX	NATURE OF TESTS	MEAN METABOLISM RATE	STANDARD DEVIATION	COEFFICIENT OF VARIATION
Totonicapan	134	M	Basal	$+8.20\pm0.958$	5.24 ± 0.674	$63.92\pm\ 8.24$
Totonicapan	20	M	3 hours after chili feeding	$+8.46\pm1.34$[1]	6.01 ± 0.949	71.03 ± 11.22
Totonicapan	12	M	3 hours after tortilla feeding	$+1.30\pm2.10$	7.29 ± 1.48	58.90 ± 12.02
Samayac laborers	30	M	Basal	$+5.20\pm1.14$	6.26 ± 0.808	$120\ \ \pm15.5$
White	2	F	Basal	−6		
White	6	M	Basal	-11.5 ± 1.21	2.98 ± 0.861	$30.84\pm\ 8.90$

[1] Increase over subjects' own basal rate.

individuals of similar sex, age, weight and height. The mean value of $+8.20 \pm 0.958\%$ secured on the soldiers, we take to be a close approach to the true basal metabolic rate for this group. The tests were run at from 11.5 to 16°C. open

[1] The gauge of this apparatus delivers a constant volume of oxygen under varying degrees of temperature and pressure. In repeated comparative tests at varying pressures with a water spirometer we have found the variations in metabolism readings to run less than 3%. We have measured the accuracy of the machine at barometric pressures as low as 54.5 mm. of Hg. In repeated tests we find that the gauge measures within 1% the volume of the gas as measured under standard conditions with the water spirometer.

air temperatures. Since the subjects were well blanketed and appeared comfortable at all times, we believe that the outside temperature did not increase their basal metabolic rate. Apparently this temperature is within their habitat temperature range. By habitat temperature we include that temperature range to which the individual or the animal is normally subjected and apparently adapted. The barometric pressure which averaged 506 mm. of Hg. for six consecutive daily readings apparently does not affect the basal metabolic rate to any extent for the tests on the laborers at 800 feet altitude averaged $+5.20 \pm 1.14\%$. Five tests under basal conditions upon one white female and one white male subject at 8100 feet altitude gave an average value of -7.5%. Three tests upon another white male at 800 feet gave a value of -10%.

The twenty tests made 3 hours after chili feeding showed an average percentage increase over the subject's own basal rates of $+8.46\%$ while tortilla feeding increased the rate by $+1.30\%$.[2]

The average values for both the Maya soldiers and laborers are in keeping with the values of Benedict ('29) of $+5.2\%$ on thirty-two male Mayas at Chichin Itza Yucatan, secured by Williams and Benedict ('29) and with those of Shattuck and Benedict ('30) viz., $+5.8\%$ on thirty Indian subjects. Steggerda ('32) verified these earlier findings and secured an average of $+8\%$ above normal white metabolism standards.

It appears from the findings of MacLeod, Earle, Necheles, Heinbecker, Okada, Van Berkhout, to mention only a few workers in addition to those already cited, that definite racial differences in basal metabolic rates exist. A striking fact, however, is that the Indian of Yucatan, Guatemala and

[2] Recently we have tested the effect of chili on a number of guinea pigs and human subjects. Our maximum dosage was 1½ gm. of dry chili for the human subject and up to ½ gm. for the guinea pigs. These tests on the human subjects have given negative results to date. In the case of the guinea pigs, our first tests were negative but continued dosage over about 3 weeks increased the basal metabolic rate from 12 to 14% in some cases. It is possible that continued use of chili might have a stimulatory effect on the thyroid glands since there is evidence of hyperplastic change in some of our treated pigs.

the Navajo [3] of Arizona and the Eskimo, all run counter to the findings made on the Mongolians of China, Japan and Java.

BLOOD PRESSURES AND PULSE RATES

The blood pressure on the soldiers averaged 111 systolic and 77 diastolic, on the laborers it averaged 104 and 73. The average for the white subjects with an average age of 31.6 years was 126 systolic and 74 diastolic. The pulse rates on the soldiers ran 63 before and 61 after the tests while those of the laborers ran 62 before and 60 after. The white female had a pulse rate of 72 while the white males averaged 68.

GOITER

We have observed a high incidence of diffuse endemic goiter throughout the highland regions. All the Indian subjects with the exception of nos. 22 and 26 were examined by Doctor Crile for goiter, only in no. 31 was no evidence of goiter found.

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[3] In a personal communication from Dr. C. G. Salsbury of the Sage Memorial Hospital, Ganado, Arizona, he states, "We have recently compiled figures on 1136 basal metabolisms (Navajos) and these show an average of +2.4%; 1277 blood pressures show a systolic of 111.9 and a diastolic of 71.6."

THE RELATION OF VITAMIN C DEFICIENCY TO NUTRITIONAL ANEMIA [1]

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ONE FIGURE

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The occurrence of anemia in scurvy has been recognized for a long time. However, the degree of anemia very rarely parallels the other symptoms which are regarded as characteristic for scurvy (Hess and Fish, '14; Hess, '20; Aron, '22; Mettier, Minot and Townsend, '30; Rohmer and Bindschedler, '32; and Parsons and Smallwood, '35).

The frequent and simultaneous occurrence of anemia and scurvy has an important bearing on the cause of nutritional anemia. A study by Aron ('27) of the cases of nutritional anemia treated between 1908 and 1926 in the University Children's Hospital at Breslau revealed that during the period of frightful shortage of food material, 1921 to 1923, there was an extraordinary increase in the number of cases admitted for severe anemia coincident with a hitherto unknown number of cases of scurvy observed during the same period. Fifteen cases of anemia were carefully examined for scurvy according to clinical symptoms and x-ray findings. Such symptoms were found in seven instances (Aron, '22). It is remarkable that Rohmer and Bindschedler ('32), recording the results of blood examinations in fifteen cases of infantile scurvy, found anemia in 'only seven instances.'

Before ascorbic acid was discovered only so-called 'antiscorbutic foodstuffs,' like orange, lemon, or other fruit juices,

[1] This investigation was conducted under a grant from the Emergency Committee in Aid to Displaced Foreign Medical Scientists.

green vegetables or germinated oats, all of them containing a number of other constituents, could be used in clinical or experimental investigations. Therefore, it was not possible to decide which constituent of the so-called 'antiscorbutics' was the active factor causing anemia by deficiency and acting as a hemopoietic when added to the diet.

After vitamin C became available in chemically pure form as ascorbic acid, some investigations were made concerning its influence upon the blood, especially upon the red blood cells and reticulocytes. However, the results reported thus far are contradictory in several respects and the fundamental problems are not satisfactorily solved (Jonas, '23; Meyer and McCormick, '28; Mettier, Minot and Townsend, '30; Mettier and Chew, '32; Rohmer and Bindschedler, '32; Dunlop and Scarborough, '35; Parsons and Smallwood, '35; Euler and Malmberg, '36, '37, '38; Aszodi, '37; Kenney and Rapoport, '38; Mettier, '38, and Ungley, '38).

Since the fundamental problem of the relationship of ascorbic acid to blood formation and to anemia cannot be solved by clinical studies alone, a systematic experimental investigation is necessary. There are very detailed procedures and rigid rules for the study of the different scorbutic symptoms in experimental scurvy of guinea pigs. These include a study of the body weight, the post mortem findings on the various organs, the bones and even the histological structure of the teeth. However, until now no attempt has been made to include a study of the hemoglobin and the blood cells in the 'biological assay' for vitamin C. It is obvious that for the purpose of 'biological assay' such blood examination if applicable would offer certain advantages over methods based only on post mortem findings or histological examination.

EXPERIMENTAL PROCEDURE

a. First series. In the first series of experiments the method for biological standardization of vitamin C was strictly followed (Coward, '36, '38; King, '38).

The scorbutogenic diet (diet sc.) used throughout all experiments was prepared every 10 to 20 days by mixing the following components:

Heated skimmed milk powder [2]	300
Ground whole oats	590
Melted butterfat, strained	90
Cod liver oil	10
NaCl	10

In addition to diet sc., 5 gm. dry oats were given daily in the first and second series of experiments.

Four male guinea pigs (nos. 3, 4, 11 and 12) somewhat over 200 gm. in body weight were kept on a diet of oats and carrots for a preliminary period. Hemoglobin and red blood cells were checked at least twice. When the animals had reached weights of 265, 275, 280 and 300 gm. respectively they were transfered to the scorbutogenic diet.

The scorbutogenic diet produced the well-known signs of scurvy. A distinct reduction of hemoglobin with a slight reduction of the R.B.C., however, developed in only one of these animals. The other three animals showed no distinct anemia in spite of the fact that they had lost nearly 25% of their body weight. None of those four animals could be saved even by the administration of ascorbic acid subcutaneously or orally in doses of 25 mg. daily. These experiments agree fully with those performed by Dr. M. I. Pierce [3] in this laboratory.

From earlier experiments (Aron, '22, and Jonas, '23) we had the impression that young guinea pigs or guinea pigs of lower body weight do not become anemic as readily as do older ones of higher body weight. A critical analysis of the experiments published by Meyer and McCormick ('28) seemed

[2] The skimmed milk powder was heated in a very thin layer in flat pans for at least 4 hours. During the heating process the powder was stirred and turned over several times. The heating was continued until the milk powder showed a pronounced brown color. The purpose of this was to destroy completely vitamin C and such other heat labile factors as might be present, as e.g. the 'grass juice factor' (Koehler, Elvehjem and Hart, '38).

[3] Unpublished results.

to confirm this belief. For this reason in the next series of experiments the guinea pigs were not put on the deficiency diet before they had grown to about 500 gm. and were at least 4 months of age.

b. Second series. This experiment included six guinea pigs, all of them male (nos. 1, 2, 5, 7, 6, 10). In a preliminary period carrots and oats with milk were given. Then the animals were transfered to the scorbutogenic diet which for a limited period was supplemented either by addition of 5 gm. fresh germinated oats [4] daily, or by 12.5 mg. ascorbic acid [5] dissolved in 2 to 3 cc. of water and fed orally.

On the one hundred and first day the administration of vitamin C was discontinued and dry oats was substituted for germinated. Following this change a distinct anemia was produced in all six animals as will be seen from table 1. All these animals were in the ultimate stage of vitamin C deficiency. When we attempted to cure the anemia even amounts up to 100 mg. ascorbic acid, given either by mouth or by subcutaneous injection, did not produce a remission of the anemia. Four animals, nos. 1, 2, 5 and 7 succumbed within 3 to 6 days. However, pigs nos. 6 and 10 which on the fourth day were placed on a diet of germinated oats and concentrated milk could be saved.

In spite of the fact that the quantity of ascorbic acid contained in the germinated oats was less than 5 mg. there was a marked improvement immediately after this change of diet was introduced. After the animals had been on this supplement of 5 gm. germinated oats for a period of 10 days, the germinated oats were replaced by ascorbic acid. At first 25 mg., later 2 mg. daily were fed orally to each animal.

Before the guinea pigs had again reached their original weight the hemoglobin content of the blood was within normal limits.

[4] Five grams germinated oats, according to our chemical determination, have an average content of about 2 mg. ascorbic acid.

[5] We wish to express our appreciation to Merck and Co., Rahway, N. J., for furnishing the ascorbic acid (Cebione) used in this investigation.

c. A third series of experiments was initiated with the intention of starting the supplement with the anemic animals before they reached the ultimate (fatal) stage. Six male guinea pigs all of whom had been watched closely for more than 100 days were fed our scorbutogenic diet (diet sc.) with an addition of either ascorbic acid (Cebione) or a juice pressed from germinated oats for 53 to 68 days respectively.

TABLE 1

NUMBER OF ANIMAL	SUPPLEMENT TO DIET SC.	AVERAGE BEFORE WITHDRAWAL OF ANTISCORBUTIC	LOWEST VALUE AFTER WITHDRAWAL OF ANTISCORBUTIC
1	68th to 100th day: 5 gm. dry oats +12.5 mg. ascorbic acid	Hgb. gms. 14.9	8.2
		Ery. mill. 4.5	2.2
2	68th to 100th day: 5 gm. dry oats +12.5 mg. ascorbic acid	Hgb. gms. 14.8	12.4
		Ery. mill. 4.6	3.7
6	82nd to 100th day: 5 gm. dry oats +12.5 mg. ascorbic acid	Hgb gms. 14.2	11.1
10	82nd to 100th day: 5 gm. dry oats +12.5 mg. ascorbic acid	Hgb. gms. 14.5	10.6
5	68th to 100th day: 5 gm. germinated oats	Hgb. gms. 15.4	11.6
		Ery. mill. 4.9	4.3
7	68th to 100th day: 5 gm. germinated oats	Hgb. gms. 15.9	11.8
		Ery. mill. 4.8	4.3
Average	Hemoglobin, grams	14.9	11.0 = 26% reduction
	Erythrocytes, mill.	4.7	3.7 = 21% reduction

The life duration of a red blood cell hardly amounts to more than 50 days. Consequently we are fully justified in stating that the red blood cells of these guinea pigs and therefore the hemoglobin within these cells was built up entirely while these animals were on diet sc. with the respective supplement.

As the anemia produced by the withdrawal of vitamin C is always of a distinctly hypochromic type, the decrease in

hemoglobin is comparatively greater than that in the number of red blood cells (see also table 1). For this reason it seemed highly important to concentrate our studies upon the hemoglobin changes. In order to make the hemoglobin determinations as accurate as possible they were performed in duplicate with two different types of hemoglobinometers, the one checking against the other (Newcomer and Hellige or Sahli Leitz). Blood was drawn at intervals from 2 to 5 days.

In guinea pigs nos. 6 and 10 the withdrawal of the ascorbic acid supplement produced anemia for a second time; the loss of hemoglobin amounting to 24% and 30% respectively.[6] No attempt was made to cure these animals which were made anemic for a second time.

With the other four animals of this group, however, we gave the ascorbic acid anew in sufficient time to cure the animals. Once or twice daily 50 mg. of ascorbic acid were given either subcutaneously[7] or orally. With these large doses all four animals were saved.

Following this administration of ascorbic acid, a surprisingly fast regeneration of hemoglobin was observed. When the weight curves began to rise, the hemoglobin levels had regained their normal values. The most important figures are compiled in figure 1.

Throughout all experiments it was noticed that the more the anemia develops the more difficult it becomes to obtain sufficient amounts of blood for hemoglobin determinations by pricking the ears of the guinea pigs. However, within a few days after ascorbic acid is given anew, there is again ample bleeding from the same ears from which a short time before, with exactly the same technic, blood could be obtained only with great difficulty. This may be due to a reduction of the total

[6] These animals in addition to the scorbutogenic diet were given an extra supplement of iron from the time the first decline in hemoglobin was noticed. Iron pyrophosphate in doses of 0.2 to 0.5 gm. daily were given in the drinking water. This mode of iron administration was adopted since the animals while declining in weight as well as hemoglobin still drank some water, while the food was hardly touched.

[7] 'Cenolate' kindly furnished by Abbott Laboratories, North Chicago, Ill.

blood volume. It may also be due to desiccation. Such desiccation would explain why some investigators have reported an increase of red blood cells and of hemoglobin in clinical as well as in experimental scurvy (Hess and Fish, '14; Hess, '20 and Aszodi, '37). In our experiments there was also a slight rise noted in some instances after the lowest values for hemoglobin were obtained.

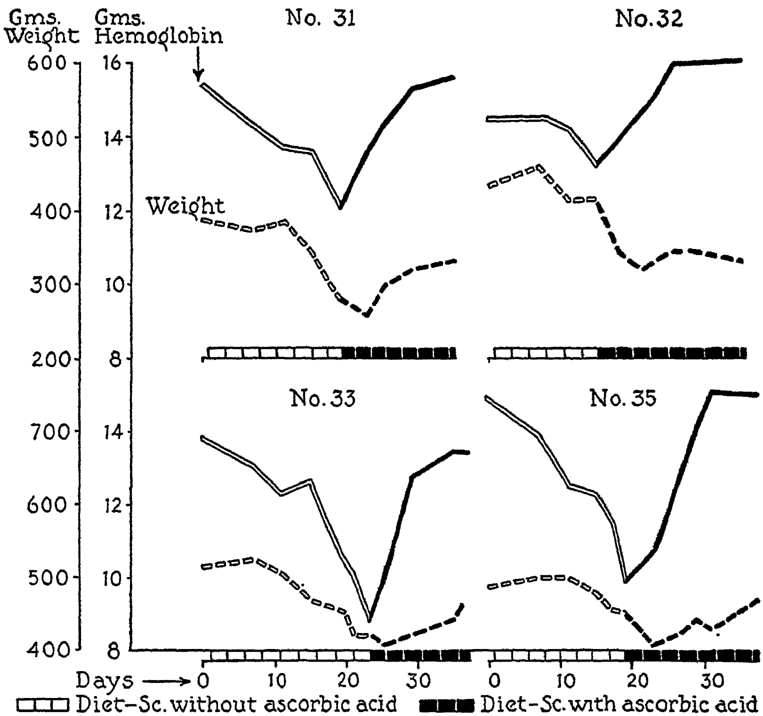

Fig. 1 For each graph: Upper curve: Hemoglobin. Lower curve: Weight.

SUMMARY

1. When guinea pigs are fed a scorbutogenic diet with an addition of ascorbic acid for 50 days or more, a period longer than the life cycle of the erythrocyte, normal blood formation takes place.

2. When the ascorbic acid supplement is withdrawn, guinea pigs 4 months of age having a body weight of 450 gm. or more

show a distinct reduction in the hemoglobin content of the blood within 20 days. A supplement of iron does not prevent this decline in hemoglobin. Younger animals, of 200 to 300 gm. body weight, may also become anemic. However, they very rarely do so because they usually succumb before a distinct anemia develops.

3. Guinea pigs made anemic by the withdrawal of ascorbic acid from their diet can be cured by administration of ascorbic acid in large amounts either orally or subcutaneously. This cure, however, is successful only in animals which have lost not more than about 25% of their body weight or one-third of their hemoglobin. The rise in hemoglobin induced by the ascorbic acid medication takes place much faster than the rise in body weight. This indicates that the anemia is cured long before the repair of the other body tissues is accomplished.

4. While these experiments give unquestionable evidence that ascorbic acid is a factor of deciding influence on hemoglobin formation in the guinea pig, they do not prove that ascorbic acid is the only active factor present in fresh green vegetables or in germinated oats as used in this experiment.

The problem of comparing the activity of ascorbic acid present in fresh green foods with the ascorbic acid in chemically pure form will be taken up in another paper.

I am deeply indebted to Prof. Chester J. Farmer for his advise, cooperation and his courtesy at all times. I also wish to thank Mr. W. L. Lummis for technical assistance.

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UTILIZATION OF ENERGY OF WHEAT PRODUCTS BY CHICKENS [1]

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The energy values of feeds and foods were formerly compared on the basis of the chemical analyses for protein, ether extract, crude fiber and nitrogen-free extract. Next in point of time they were compared on the basis of digestible nutrients, with the assumption that the digestible nutrients of the same class have the same value to the animal, regardless of origin. This procedure is still being used (Morrison, '36), though experimental evidence with cattle is conclusive that such equality often does not exist and that the digestible constituents of some feeds, such as roughages, sometimes have a value of half or less of the digestible constituents of others, such as concentrates. The calories of human foods are now usually calculated (Morey, '36; Rose, '38; Sherman, '37) by multiplying the grams of protein by 4, the fat by 9 and the carbohydrates (which sometimes includes the crude fiber) by 4. The calories for human food obtained by such calculations, sometimes called the physiological fuel value (Sherman, '36), are presumably the metabolizable energy, which is the total energy less the energy lost in the solid and liquid excrements. This method of calculating the energy values of human foods was devised for a mixed diet (Morey, '36) and when applied to single foods incorrectly assumes that all foods are equally digestible and also assumes that the energy values of the metabolizable energy of different feeds are equal. As will be shown in this paper, this assumption is also incorrect.

[1] Read at the Baltimore meeting of the American Chemical Society, April 6, 1939.

The energy values of feeds, as measured by the energy of the fat and flesh which can be produced on fattening cattle when the feeds are added to a diet already sufficient for maintenance, have been extensively studied and for a long time have been used for comparing energy values of cattle feeds, formulating feeding standards, etc. (Armsby, '17; Forbes and Kriss, '25; Fraps, '11, '25, '28; Kellner, '05). Such energy values, expressed in therms per 100 pounds of feed, have been called the productive energy by Fraps and the net energy by Armsby and by Forbes. Only a few estimates of the productive energy of chicken feeds have been made. Mitchell and Haines ('37) estimated the net energy of corn for chickens to be 2.83 calories per gram, Southgate ('29) calculated that a mixture of 13 parts oats and 3 parts dried whole milk has a net energy of 1.8 calories per gram, and Fraps and Carlyle ('39) found the net energy of a ration for chickens to be 1.79 calories per gram. The work here reported is a comparison of the productive energy values of patent flour, low-grade flour, wheat bran and wheat brown shorts, with cornmeal as a standard as measured by the energy of the protein and fat gained by young growing chickens.

METHODS

Four groups of six chickens each were used in the tests. One group was fed a ration containing 50% of cornmeal, while the other three were fed rations containing 50% of the feed to be tested. The remainder of the ration was the same in all four groups and all the other conditions were made as uniform as possible, so that the only variable was the feed being tested. The basal 50% of the ration consisted of 20% wheat brown shorts, 12% casein, 6% cornmeal, 6% alfalfa leaf meal, 2% yeast, 1% calcium carbonate, 1% salt, 1% tricalcium phosphate and 1% of fortified fish oil. Sixty or more baby chicks were fed in a preliminary period of 1 week on the cornmeal ration, and then four groups of six each were selected so as to have the same average weight. Four of the remaining chicks, as nearly similar to the others as possible, were ana-

lyzed at the beginning of the experiment, and the other remaining chicks were used for digestion experiments on the rations being studied by methods previously used (Fraps, '28). The experimental chicks were placed in individual compartments in battery brooders, fed individually for 3 weeks, and weighed at the end of each week. They were then killed, the intestinal contents removed, and the entire chick ground up together with about 1% boric acid and 3% filter paper pulp. Protein (N $\times$ 6.25) and fat were determined at

TABLE 1

Percentage composition of feeds

	EXPERIMENT NUMBER	PROTEIN	ETHER EXTRACT	CRUDE FIBER	NITROGEN FREE EXTRACT	WATER	ASH
Cornmeal	6–6	11.05	3.00	1.39	73.15	9.93	1.48
Cornmeal	6–7	9.86	4.11	1.36	72.78	10.84	1.05
Cornmeal	6–8	11.88	4.96	1.58	69.63	10.27	1.68
Patent flour	6–6	12.57	0.91	0.25	73.25	12.55	0.47
Patent flour	6–7	12.83	0.84	0.24	73.43	12.17	0.49
Patent flour	6–8	13.96	1.01	0.40	70.88	13.22	0.53
Low-grade flour	6–6	13.20	2.24	0.43	71.67	11.52	0.94
Low-grade flour	6–7	15.07	1.94	0.50	70.18	11.46	0.85
Low-grade flour	6–8	18.78	2.08	0.50	64.89	12.74	1.01
Wheat bran	6–6	17.87	4.18	10.13	52.47	8.93	6.42
Wheat bran	6–7	19.24	4.05	9.41	50.64	10.33	6.33
Wheat brown shorts	6–8	19.03	4.24	6.43	54.83	10.51	4.96
Wheat brown shorts	6–6	19.33	4.77	6.69	54.95	9.53	4.73
Wheat brown shorts	6–8, 6–7	19.50	4.62	6.47	54.94	9.57	4.90

once, not less than three determinations being made on each chick. After correcting for additions of boric acid and paper pulp, the energy content was calculated by use of the factors 5.66 for protein and 9.35 for fat. This method was found by Fraps and Carlyle ('39), to give results agreeing with the heats of combustion determined in a bomb calorimeter.

RESULTS OF WORK

Three experiments are here reported. The composition of the feeds used is given in table 1. Analyses of the rations

also were made and had practically the same composition as those calculated from the analyses in table 1 and the percentages of the ingredients.

The weights and average composition of the chicks and other data for the three experiments are given in table 2. At

TABLE 2

Composition and weights of chickens

	LIVE WEIGHT AT BEGINNING	LIVE WEIGHT AT END	EMPTY WEIGHT AT END	PROTEIN	FAT	CALORIES PER 100 GM. EMPTY WEIGHT
	gm.	*gm.*	*gm.*	%	%	
Beginning of experiment 6–6	65.5	65.5	56.3	16.74	7.42	164.1
Calories per 100 gm. live weight 141.2						
Cornmeal ration	65.9	191.7	181.4	20.09	8.81	196.1
Patent flour ration	65.6	187.6	169.7	20.14	7.75	186.4
Low-grade flour ration	65.9	181.6	173.8	20.34	6.15	172.6
Wheat bran ration	65.8	177.2	167.9	20.84	2.96	145.6
Beginning of experiment 6–7	51.5	51.5	49.5	16.93	5.23	144.7
Calories per 100 gm. live weight 139.2						
Cornmeal ration	53.7	167.7	163.8	20.32	8.37	192.4
Patent flour ration	52.9	161.1	156.9	20.93	6.13	175.8
Low-grade flour ration	52.9	173.4	169.6	21.25	6.23	178.5
Wheat bran ration	53.1	159.0	152.2	21.70	2.83	149.2
Beginning of experiment 6–8	59.9	59.9	58.2	18.17	6.79	166.3
Calories per 100 gm. live weight 161.4						
Cornmeal ration	60.5	209.7	203.2	20.34	8.04	190.3
Patent flour ration	59.6	194.7	189.7	20.93	7.04	184.3
Low-grade flour ration	59.9	201.0	194.7	20.79	5.04	164.8
Wheat brown shorts ration	60.4	199.9	193.9	21.03	3.70	153.7

the end of the experiment the chicks fed the wheat bran ration averaged only about 3% fat and those fed on the wheat brown shorts ration averaged only 3.7% fat as compared with more than 8% fat in the chickens fed on the cornmeal ration. There are great differences in fat and energy content for the different rations. The live weights at the end of the experiment

averaged 191.7 gm. for the cornmeal ration, compared with 177.2 for the wheat bran ration in experiment 6, and 167.7 gm. compared with 159.0 in experiment 7. The energy value of the ration may thus affect the fatness of the chickens more than it affects the live weight.

The results of the chemical analyses, the digestion experiments and the estimation of metabolizable energy are summarized in table 3, both for the rations and for the feeds.

TABLE 3

Effective organic nutrients, effective digestible nutrients and metabolizable energy per 100 gm. of rations and of feeds

		RATIONS			FEEDS		
		Effective organic constituents	Effective digestible nutrients	Metabolizable energy	Effective organic constituents	Effective digestible nutrients	Metabolizable energy
		gm.	*gm.*	*cal.*	*gm.*	*gm.*	*cal.*
Basal part of ration		82.0	59.0	274.0	..	..	..
Cornmeal	Exp. 6	86.4	70.5	319.3	91.0	82.0	364.6
Patent flour	Exp. 6	85.8	68.5	307.0	87.9	78.0	340.0
Low-grade flour	Exp. 6	86.5	66.6	299.9	89.9	74.2	325.8
Wheat bran		83.2	47.9	213.1	79.8	36.8	152.2
Cornmeal	Exp. 7	86.9	68.0	322.3	91.9	77.0	370.0
Patent flour	Exp. 7	86.5	69.4	311.7	88.2	79.8	349.4
Low-grade flour	Exp. 7	85.8	68.3	313.0	89.6	77.6	352.0
Wheat bran	Exp. 7	80.8	45.9	208.3	79.0	32.8	142.6
Cornmeal	Exp. 8	88.6	71.2	320.4	92.7	83.4	366.8
Patent flour	Exp. 8	86.8	69.9	319.1	87.1	80.8	364.2
Low-grade flour	Exp. 8	85.6	65.2	293.2	88.4	71.4	312.4
Wheat brown shorts	Exp. 8	83.6	53.5	241.8	83.4	48.0	209.6

The effective organic constituents are the sum of the protein, the ether extract multiplied by 2.25, and the nitrogen-free extract, as found in the analyses made of the rations and of the feeds. The crude fiber is considered to have no energy value for chickens. The effective digestible nutrients are the sum of the digestible protein, the digestible ether extract multiplied by 2.25 and the digestible nitrogen-free extract. For the rations they were calculated directly from the average data of two digestion experiments. For 100 parts of the feeds

they were obtained by deducting the effective digestible nutrients of one-half of the basal part of the ration from those of the total ration and multiplying by 2. For example, for cornmeal in experiment 6, 59.0 divided by 2 and the result subtracted from 70.5 and multiplied by 2 gives 82.0, the effective digestible nutrients in 100 parts of the cornmeal. The metabolizable energy is the gross energy of the feed minus the energy of the excrement, since with the chickens the excrement contains the urinary excretion as well as the undigested material. The metabolizable energy of the rations was determined in connection with the digestion experiments and that of 100 parts of the feeds by subtracting one-half the

TABLE 4

Maintenance requirements calculated from the cornmeal ration

EXPERIMENT NUMBER	AVERAGE WEIGHT OF CHICKS BY PERIODS	INITIAL ENERGY CONTENT OF CHICKS	FINAL ENERGY CONTENT OF CHICKS	GAIN OF ENERGY OF CHICKS	PROD. ENERGY OF RATION	TOTAL RATION EATEN	PROD. ENERGY OF FEED EATEN	FOR MAINTENANCE	
								Total prod. energy	Prod. energy per period and 100 gm. chicken
	gm.	*cal.*	*cal.*	*cal.*	*cal. per gram*	*gm.*	*cal.*	*cal.*	*cal.*
6	118.0	93.1	357.5	264.4	1.96	275.2	539.4	275.0	234.5
7	107.3	74.7	315.5	240.8	1.89	249.0	470.6	229.8	215.2
8	131.9	97.6	386.8	289.2	1.98	288.6	571.4	282.2	214.2

metabolizable energy of the basal part of the ration and multiplying by 2 as was done with the effective digestible nutrients. The determinations of heats of combustion for this work were made in an Emerson bomb calorimeter by Dr. J. F. Fudge.

Data for the calculations for the maintenance requirements and the value of the rations for fattening are given in tables 4 and 5. The maintenance requirements are calculated with use of the average weights by periods. The average weight by periods is the average of the average weight for each of the 3 weeks, secured by the formula $\frac{a+b}{2} + \frac{b+c}{2} + \frac{c+d}{2}$, divided by 3, in which a is the live weight at the beginning, and b, c,

and d, the weights at the end of the first, second and third week respectively. The use of the average weight by periods has been shown by Fraps and Carlyle ('31) to give more consistent results that the use of the average of the first and last weights. The use of the weight as a basis for calculating the maintenance requirements was also shown by Fraps

TABLE 5

Calculation of productive energy of rations

	EXPERIMENT NUMBER	AVERAGE WEIGHT OF CHICKS BY PERIODS	INITIAL ENERGY CONTENT OF CHICKS	FINAL ENERGY CONTENT OF CHICKS	GAIN OF ENERGY OF CHICKS	RATION EATEN	USED FOR MAINTENANCE	FOR GAIN AND MAINTENANCE	PRODUCTIVE ENERGY OF RATION
		gm.	*cal.*	*cal.*	*cal.*	*gm.*	*cal.*	*cal.*	*Calories per 100 gm.*
Patent flour ration	6	111.6	92.6	316.2	223.6	271.9	262.2	485.8	179.2
Low-grade flour ration	6	115.4	93.1	302.1	209.1	277.2	271.1	480.2	173.7
Wheat bran ration	6	114.6	93.0	244.8	151.8	356.5	268.8	420.6	117.8
Patent flour ration	7	103.8	73.6	276.6	203.0	249.0	223.3	426.2	171.8
Low-grade flour ration	7	111.6	73.6	303.4	229.8	260.6	239.9	469.6	180.0
Wheat bran ration	7	106.5	74.0	228.4	154.4	355.2	228.9	383.3	107.2
Patent flour ration	8	128.4	96.1	351.8	255.0	299.8	274.8	529.8	176.4
Low-grade flour ration	8	127.7	96.7	321.6	224.9	289.2	273.3	498.2	172.3
Wheat brown shorts ration	8	124.1	97.5	297.9	200.4	376.1	265.7	466.1	125.0

and Carlyle ('39) to give results better in accord with previous work of others than the use of the surface area.

For the purpose of comparing the energy values of the other feeds with that of cornmeal, 1 gm. of the effective digestible nutrients of the cornmeal ration and of the cornmeal consumed over maintenance was assumed to produce 2.78 calories of gain in the chicken. This is the average value secured by Fraps and Carlyle ('39) for a mixed ration con-

taining 51% cornmeal, 19% wheat brown shorts, 10% dried buttermilk, 6% cottonseed meal, 5% alfalfa leaf meal, 4% tankage, 2% bone meal, 2% oyster shell and 1% salt. As the equality of the digestible nutrients of the mixture is assumed, and as the work here reported shows that the value of the digestible nutrients of wheat brown shorts is much below that of cornmeal, the 2.78 calories per gram of digestible nutrients is too low for the cornmeal ration. However, correction of this value must await more data as to the value of the energy contained in the various ingredients of the ration used by Fraps and Carlyle ('39). Since the object of this work is to secure comparative energy values of the feeds tested, rather than absolute values, the use of too low a value for productive energy will not introduce any large error. The productive energy of the cornmeal ration given in table 4 was secured by multiplying the effective digestible nutrients of table 3 by 2.78.

Since cornmeal is used as the standard, the calories of productive energy used for maintenance under the conditions of each experiment are calculated from the cornmeal ration, as given in table 4. The initial energy content of the chicks is calculated from the initial live weight and the initial energy per gram, as found by analysis (table 2) and the final energy content from the final empty weight and final energy content of the chicks. The productive energy of the ration eaten is the grams eaten multiplied by the productive energy (1.96 in case of experiment 6). The productive energy used for maintenance is the productive energy of the ration eaten less the gain of energy in calories, of the chicks, since by definition, the productive energy is measured by the gain in energy. The maintenance requirements are calculated to calories of productive energy required to maintain 100 gm. of chicken for the period of each experiment.

The maintenance requirements calculated in table 4 are used in table 5 to calculate the productive energy of the rations. The method of procedure should be obvious from the headings of the table. The sum of the calories of productive

energy used for gain and for maintenance divided by the quantity of feed eaten and multiplied by 100 gives the calories of productive energy of 100 gm. of the ration, and these are given in the last column of table 5.

TABLE 6

Comparative productive energy of feeds in calories

EXPERIMENT NUMBER	CORNMEAL	PATENT FLOUR	LOW-GRADE FLOUR	WHEAT BRAN	WHEAT BROWN SHORTS
	Productive energy per 100 gm. of feed				
6	228	194	183	71	..
7	214	179	196	50	..
8	232	189	181	..	86
Average	225	188	187	61	86
	Productive energy per 100 gm. of effective organic constituents				
6	251	221	204	89	..
7	233	203	219	63	..
8	260	217	205	..	103
Average	248	214	209	76	103
	Productive energy per 100 gm. of effective digestible nutrients				
6	278	249	247	193	..
7	278	224	253	152	..
8	278	234	254	..	179
Average	278	236	251	173	179
	Productive energy per 100 calories of metabolizable energy				
6	63	57	56	47	..
7	58	51	56	35	..
8	63	52	58	..	41
Average	61	53	57	41	41

The averages given in tables 5 and 6 are calculated from the data for individual chickens. The results would be slightly different had average data been used in the calculating of the maintenance requirements or of the productive energy.

The difference between the assumed productive energy value of 100 gm. of the corn meal ration (196 cal.) and of

the patent flour ration (179) gives the effect (—17) of substitution of 50 gm. of patent flour for 50 gm. of cornmeal. Twice this difference (—34) added to the productive energy of the cornmeal (228) gives the productive energy of 100 gm. of the patent flour (194 cal.). The productive energy values of the other feeds were calculated in a similar way.

TABLE 7

Productive energy of feeds compared with cornmeal as 100

EXPERIMENT NUMBER	CORNMEAL	PATENT FLOUR	LOW-GRADE FLOUR	WHEAT BRAN	WHEAT BROWN SHORTS
Productive energy per 100 gm. of feed					
6	100	85	80	31	..
7	100	84	92	23	..
8	100	81	78	..	37
Average		83	83	27	37
Productive energy per 100 gm. of effective organic constituents					
6	100	88	81	35	..
7	100	87	94	27	..
8	100	83	79	..	40
Average		86	85	31	40
Productive energy per 100 gm. of effective digestible nutrients					
6	100	90	89	69	..
7	100	81	91	55	..
8	100	84	91	..	64
Average		85	90	62	64
Productive energy per 100 calories of metabolizable energy					
6	100	90	89	75	..
7	100	88	97	60	..
8	100	83	92	..	65
Average		87	93	68	65

The productive energy values of the feeds are given in the top section of table 6. By means of these data and the data in table 3 calculations have been made for the productive energy in calories per 100 gm. of effective organic constituents, of effective digestible nutrients and of 100 calories of metabolizable energy.

DISCUSSION OF RESULTS

With the average productive energy of cornmeal at 225 calories per 100 gm., the average productive energy of the patent flour as given in table 6 is 188 calories, that of the low-grade flour is 187, that of the wheat brown shorts is 86 and that of the wheat bran is 61. The productive energy of wheat bran is a little more than one-fourth of that of cornmeal. The average productive energy per 100 gm. effective digestible nutrients are the assumed value of 278 for the cornmeal, 236 for the patent flour, 173 for the wheat bran and 179 for the wheat brown shorts. That is, the energy value per gram of digestible constituents differs in different feeds and the quantity of digestible constituents is not a correct measure of the value of a chicken feed for growth. It is also seen in table 6 that 61% of the calories of metabolizable energy are utilized from cornmeal but only 41% from wheat bran or wheat brown shorts. The metabolizable energy is not a correct measure of the value of a feed for growth and production.

Compared with that of cornmeal at 278 calories the productive energy per 100 gm. of digestible nutrients of the patent flour averages 236 and that of the low-grade flour 251. It is impossible at the present time to say why the digestible nutrients of flour have lower productive energy than cornmeal. The chemical compounds (chiefly starch and protein) do not appear to be sufficiently dissimilar in quantity or quality to cause these differences. The possibility of a vitamin deficiency in the rations is being investigated.

The productive energy value per unit of the digestible nutrients of the wheat brown shorts and of wheat bran are 64% and 62% of those of cornmeal. These lower values perhaps may be partly accounted for on the assumption that the products of the digestion of the pentosans and hemicelluloses present in wheat bran and wheat brown shorts are not as completely utilized by the animal body as the sugars formed from the digestion of the starches of cornmeal and wheat flour. This explanation, of course, requires further study.

The relative productive energy of the metabolizable energy of low-grade flour is 57, of patent flour is 53, of wheat brown shorts is 41 and of wheat bran is 41, compared with that of cornmeal at 61. The metabolizable energy is a little better measure of productive energy than the digestible nutrients but not sufficiently accurate for use. When measured by gains of protein and fat on growing chickens, neither the total nutrients, the digestible nutrients, nor the metabolizable energy are correct measures for the relative values of cornmeal, wheat flour, wheat brown shorts and wheat bran. Since the difference between the productive energy and the metabolizable energy must be dissipated as heat, wheat bran and wheat brown shorts should produce a greater excess of heat to dissipate than cornmeal or wheat flour, and this fact may be especially important in warm climates.

The human digestive organs are different from those of chickens, and the quantity of material digested by a human from a given food may be different from that digested by a chicken from the same food. However, once the food has been digested, there may be little relative difference in utilization of the energy of the digested materials. The results here presented at least indicate that wheat bran may have a low value in human food and that the removal of the bran (about 18% of the whole wheat grain) and of wheat brown shorts (about 12%) in the process of flour milling may be an advantage. They indicate that graham flour or whole wheat flour may have about 90% of the energy value of white flour. They indicate that cornmeal, wheat flour, graham flour and whole wheat flour may differ in productive energy for human food and not be equal in energy value as is at present assumed to be the case (Morey, '36; Sherman, '37; Rose, '38) and that the relative energy values now used need to be studied and revised.

SUMMARY

The value of several feeds for maintenance and for production of gain when fed to young growing chicks was compared with the value of cornmeal. The feeds to be compared

were fed as half of a ration otherwise constant. The energy content was calculated from analyses made for protein and fat on representative chicks at the beginning and the chicks on experiment after 3 weeks of the feeding tests. The maintenance requirements were calculated from the data secured with the cornmeal ration. The calories of energy gained were calculated from the data of the other tests made at the same time. With the cornmeal ration as a standard, three tests were made with patent flour and low-grade flour, two with wheat bran and one with cornmeal. With the productive energy of cornmeal at 225 calories per 100 gm., the relative productive energy values of the other feeds averaged: patent flour 188, low grade flour 187, wheat brown shorts 86 and wheat bran 61. The relative productive energy values of effective digestible nutrients averaged, with cornmeal at 278, in patent flour 236, in low-grade flour 251, in wheat brown shorts 179 and in wheat bran 173. The relative productive energy values of the metabolizable energy were, with cornmeal at 61, patent flour 53, low-grade flour 57, wheat brown shorts 41 and wheat bran 41. When measured by gains of energy in protein and fat of growing chickens, neither the total nutrients, the digestible nutrients, nor the metabolizable energy are correct measures of the feeding values of cornmeal, wheat flour, wheat brown shorts and wheat bran.

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THE EFFECTS PRODUCED BY AN INCREASE IN THE CALCIUM AND PHOSPHORUS CONTENT OF THE DIET ON THE CALCIUM AND PHOSPHORUS BALANCE AND ON VARIOUS BODILY CONSTITUENTS OF THE RAT[1]

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An excess of inorganic phosphate in the diet of albino rats in the form of orthophosphoric acid, or as the acid, basic or neutral phosphate of sodium or potassium has been shown by MacKay and Oliver ('35) to result in pronounced renal lesions. Calcification of a necrotic debris that fills the tubules presented a striking feature of the lesions. These effects on the kidney which we have confirmed in preliminary experiments, suggested the present investigation in which the effects of a large intake of calcium and phosphorus on the deposition of these elements and on the weight, water, protein and fat content of the body were determined.

PROCEDURE

The animals used in these experiments were the offspring of inbred Wistar rats and were selected in groups of three litter mates of approximately the same weight. Seven groups of males and an equal number of females, or a total of forty-two animals, were used. When weaned at the age of 21 days, they were placed in individual cages and fed the Wistar diet for 7 days. During this period the food intake was controlled so that each animal consumed the same amount of food as its

[1] Presented before the American Physiological Society at the Toronto meeting, April 26–29, 1939.

litter mates. The animals were then placed on the experimental rations. The basal ration consisted of 68% sucrose, 18% casein, 10% yeast and 4% of the Osborne and Mendel salt mixture. Sufficient $CaCO_3$ was added to give a Ca/P ratio of 2:1, as determined by analysis of the mixture; without this addition the ratio was 1:1. One animal in each group was fed the basal ration to serve as a control; the other two in the group were fed the same ration with additional amounts of calcium and phosphorus. Phosphorus was added in the form of a neutral mixture of mono- and dibasic sodium phosphate in sufficient quantity to increase the phosphorus content to approximately 2.5 and 3.2 times the amount present in the basal diet. Calcium was added as $CaCO_3$ in amounts sufficient to maintain the Ca/P ratio within the range of 1.6 to 2.0. Each animal was given daily by medicine dropper, 7 drops of a 6:1 mixture of cod liver oil and linoleic acid.

The basal ration will hereafter be referred to as ration 1 and those with the larger amounts of phosphorus and calcium as rations 2 and 3, respectively. Preliminary experiments showed that on rations 2 and 3 the kidney lesions described by MacKay and Oliver occurred invariably whereas the kidneys of the control animals on ration 1 were normal.[2]

The animals were fed on the experimental rations for 10 weeks, by the paired feeding technic of Mitchell and Beadles ('30). The food intake of litter mates was kept approximately the same from day to day and exactly equalized once a week. In making this equalization the actual bulk of the food mixture consumed by the animals on rations 2 and 3, owing to the higher salt content, had to be larger than that of the controls in order that the caloric value would remain the same. The feces were collected over the entire period of the experiment including the preliminary week on the Wistar diet. The bottom of the cages was constructed of wire mesh sufficiently large to permit free passage of the feces on to a

[2] Histopathological examination of the kidneys was made by Dr. Everett Bishop, pathologist to the Steiner and the Winship Clinics, Atlanta, Georgia.

fine mesh screen beneath. The collected feces were dried, weighed and analyzed for calcium and phosphorus.

At the conclusion of the experiment the animals were fasted for 24 hours with free access to water, and then killed by a blow on the head. The bodies were chopped with a meat cleaver, ground in a sausage grinder and finally in a corn mill. The intestinal contents were not removed. This procedure produced a homogeneous mass as shown by checks obtained on analyses of several aliquots for calcium, phosphorus, fat, nitrogen and water.

The analytical procedure was as follows: An aliquot of approximately 20 gm. was boiled in 30% KOH and after boiling, one sample taken for the analysis of fat by the method of Leathes and Raper ('25) as described by Feyder ('35) and another for the determination of nitrogen by the macro-Kjeldahl method. The determinations of calcium and phosphorus were made on separate aliquots of tissue which were ashed by the usual procedure and dissolved in HCl. Phosphorus was determined by the method of the Association of Official Agricultural Chemists ('25) which was found to give approximately the same values as the Youngburg and Youngburg method ('30). Calcium was determined by the method of McCrudden as described by Peters and Van Slyke ('25). The water content of the body was determined by drying aliquots of ground tissue to constant weight over P_2O_5 in an evacuated desiccator. Loss of water in preparing the tissues was reduced to a minimum by grinding the animals in a room saturated with water vapor.

RESULTS

The data on the calcium and phosphorus balance are given in table 1 and the results of the analyses of the rats in table 2. Each value in the tables is an average obtained from seven experiments except where the males and females have been combined, in which case the averages were derived from fourteen experiments. As a general observation it may be stated that there were no sex differences discernible in the

TABLE 1

Calcium and phosphorus balance with different calcium and phosphorus intake

RATION [1]	NUMBER OF EXPERIMENTS	CALCIUM INTAKE	CALCIUM IN FECES	DIFFERENCE BETWEEN INTAKE AND OUTPUT	INCREASE IN ABSORPTION [2] OVER CONTROLS	PHOSPHORUS INTAKE	PHOSPHORUS IN FECES	DIFFERENCE BETWEEN INTAKE AND OUTPUT	INCREASE IN ABSORPTION [2] OVER CONTROLS
Males									
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	7	7.53	5.26	2.27	..	3.87	2.04	1.83	..
2	7	15.12	12.46	2.66	0.39	9.64	3.99	5.65	3.82
3	7	19.17	15.80	3.37	1.10	12.56	5.74	6.82	4.99
Females									
1	7	6.76	4.66	2.10	..	3.48	1.88	1.60	..
2	7	12.99	10.70	2.29	0.19	8.51	3.68	4.83	3.23
3	7	16.34	13.85	2.49	0.39	11.08	5.01	6.07	4.47
Males and females									
1	14	7.14	4.96	2.18	..	3.67	1.96	1.71	..
2	14	14.06	11.58	2.48	0.30	9.07	3.84	5.23	3.52
3	14	17.76	14.83	2.93	0.75	11.82	5.37	6.45	4.74

[1] Ration no. 1 contained the Osborne-Mendel salt mixture; nos. 2 and 3, 2 and 2.5 times, respectively, as much calcium and 2.5 and 3.2 times as much phosphorus as no. 1.

[2] The 'difference' in the preceding column is regarded for convenience as the amount absorbed.

results obtained from feeding the different rations except in body weight.

A statistical analysis of the body constituents is presented in table 3. The significance of the average difference between litter mates was determined by calculating the value of t according to Fisher's procedure ('28). To allow for the correlation between litter mates, t was determined by the formula on page 112 of Fisher's manual. Since the various differences between the animals on the control ration and those on rations 2 and 3 were of the same order for the males as for the females, the statistical analysis was made on the combined data for the two sexes. The probabilities of the differences obtained with different rations being a chance occurrence will hereafter be referred to as P. The values of P in the case of the body constituents are given in table 3.

The intake of calcium and phosphorus subtracted from the amount found in the feces will be referred to for convenience as the amount absorbed. It is possible that a larger amount may have been absorbed than estimated in this way, which in turn was balanced by a correspondingly larger excretion by the intestinal wall, but for present descriptive purposes it is unnecessary to take this possibility into account. With an increase in the intake of approximately 7 and 10 gm., respectively, the animals on rations 2 and 3 absorbed only 0.3 and 0.75 gm. more than those on the control diet. These increases in absorption though small were definitely significant in both cases as P was equal to 0.015 in the first instance and was less than 0.0003 in the second. With the small increase in calcium absorption on ration 2 there was a very small increase in the deposition of calcium which was not significant. On ration 3, however, with a slightly larger increase in calcium absorption there was also a slightly larger increase in deposition which, although small in absolute amount, was nevertheless significant. These results are in keeping with those of Whitcher, Booher and Sherman ('36) who found that with a phosphorus content of 0.42 to 0.43% in the diet, a graded increase in calcium from 0.43 to 1.04% did not bring about any

distinct increase in the rate of calcification in the body, but when the phosphorus content was raised to 0.73% graded increases in calcium to 1.05% induced very small increases in calcification. In our experiments the calcium content of the control ration was 1.1% and of rations 2 and 3, 2.1 and 2.6%; the phosphorus content of rations 2 and 3 was increased from 0.5% in the control ration to 1.3 and 1.7%.

Absorption of phosphorus on the different rations presents an entirely different picture than the absorption of calcium. On rations 2 and 3 with an increase of approximately 5.4 and 8.0 gm. in the phosphorus intake there was a definite increase in absorption of approximately 3.5 and 4.7 gm. (table 1). Notwithstanding this large increase in the absorption of phosphorus there was no significant change in the amount deposited in the body. Owing to the slight increase in calcium deposition without a corresponding increase in phosphorus, there was a slight rise in the Ca/P ratio of the body from 2.0 to 2.1.

The percentage gain in the body weight taken after a 24-hour fast immediately before the animals were sacrificed is given in table 2. The males gained 436, 445 and 418% over their original weight on rations 1, 2 and 3, respectively, and the females 326, 336 and 323%. This order of percentage gains was the same when the weights were taken before the fast. The small differences that were found between the groups of litter mates was in no instance significant. Conclusions drawn from these data, however, with regard to the effect of high calcium-phosphorus intake on body weight would be misleading for, as will be observed in table 2, the bodies of the animals on the high calcium-phosphorus diets contained a higher percentage of water and consequently a smaller amount of solid material than their controls. The average dry weight of the controls (males and females combined) in absolute amounts was 4.9 and 11.2 gm. more than that of their litter mates on rations 2 and 3, respectively, the differences being definitely significant (table 3). In this connection it is of interest to note that the average water

TABLE 2

Composition of rats with different calcium and phosphorus intake

RATION [1]	NUMBER OF EXPERIMENTS	INITIAL WEIGHT	FINAL WEIGHT AFTER 24-HOUR FAST	INCREASE OVER INITIAL WEIGHT	WATER CONTENT	DRY WEIGHT	FAT	PROTEIN (N × 6.25)	CALCIUM	PHOSPHORUS	Ca/P RATIO
					Males						
		gm.	gm.	%	%	gm.	gm.	gm.	gm.	gm.	
1	7	39	209	436	58.7	86.6	33.4	38.5	2.47	1.27	1.9
2	7	38	207	445	60.9	81.0	28.2	37.7	2.55	1.24	2.1
3	7	39	202	418	63.0	75.0	21.8	39.6	2.74	1.31	2.1
					Females						
1	7	38	162	326	59.8	65.0	22.8	29.8	2.30	1.10	2.1
2	7	36	157	336	61.4	60.8	19.6	29.2	2.31	1.11	2.1
3	7	35	148	323	63.6	54.2	14.2	28.3	2.34	1.12	2.1
					Males and females						
1	14	39	186	377	59.2	75.8	28.1	34.2	2.39	1.19	2.0
2	14	37	182	391	61.2	70.9	23.9	33.5	2.43	1.18	2.1
3	14	37	175	376	63.3	64.6	18.0	34.0	2.54	1.22	2.1

[1] Ration no. 1 contained the Osborne-Mendel salt mixture; nos. 2 and 3, 2 and 2.5 times, respectively, as much calcium and 2.5 and 3.2 times as much phosphorus as no. 1.

intake over the entire experiment on the three rations was 1.17, 1.64, and 2.53 liters, respectively.

The total amount of body protein was slightly different on the three rations but these differences, as shown in table 3, were not significant. The fat content, however, paralleling the decrease in dry weight, was significantly smaller with the larger intake of calcium and phosphorus. On the control ration the bodies contained 4.2 and 10.1 gm. more fat than on

TABLE 3

Statistical analysis

Comparison of body constituents on rations 1 and 2

	CALCIUM	PHOSPHORUS	DRY WEIGHT	FAT	PROTEIN
Mean difference[1]	+0.06	−0.010	− 4.9	− 4.2	−0.7
Standard deviation	0.149	0.166	4.62	4.8	2.3
Probability	0.084	0.43	0.001	0.003	0.14
Comparison of body constituents on rations 1 and 3					
Mean difference[1]	+0.17	+0.03	−11.2	−10.1	−0.2
Standard deviation	0.219	0.104	4.6	4.32	3.1
Probability	0.008	0.15	< 0.0001	< 0.0001	0.39
Comparison of body constituents on rations 2 and 3					
Mean difference[2]	+0.11	+0.04	− 6.3	− 5.9	+0.5
Standard deviation	0.226	0.107	6.2	4.1	2.4
Probability	0.052	0.10	0.001	0.0001	0.23

[1] A + sign indicates a greater average content on ration 2; a — sign the reverse.
[2] A + sign indicates a greater average content on ration 3; a — sign the reverse.

rations 2 and 3 which was practically the same as the amounts by which the controls exceeded the other animals in dry weight, namely, 4.9 and 11.2 gm., respectively. It may be concluded therefore that the smaller dry weight was due to a diminution in the formation or deposition of fat.

DISCUSSION

Since a portion of the calcium which was absorbed in rations 2 and 3 in excess of the amount absorbed in the control ration was deposited in the body, the remainder of the excess

absorbed, or 0.26 and 0.60 gm., respectively, (combined averages of males and females) must have been excreted in the urine. In the case of phosphorus, with a large increase in absorption and no increase in deposition, all the excess absorbed which amounted to 3.52 and 4.74 gm. must have been excreted.

Collection of the urine for analysis of calcium and phosphorus presented insurmountable difficulties inasmuch as the small amount of food mixture invariably spilt by the animals would have dropped into the urine thereby vitiating the results. An approximation, however, of the total urinary excretion of calcium and phosphorus on the three rations throughout the experiment can be obtained by a few simple calculations. From an analysis of three weanling male rats and three females of the same strain and approximately the same average weight as of those used in these experiments, it was found that their bodies contained 0.28 gm. calcium and 0.18 gm. phosphorus. Subtraction of these values from the total calcium and phosphorus found in the animals at the conclusion of the experiment gave 2.11, 2.15 and 2.26 gm. calcium respectively, deposited by the animals on the three rations and 1.01, 1.00 and 1.04 gm. phosphorus. The difference between the amount deposited and that absorbed (table 1) may be taken as the amount excreted in the urine. In the case of calcium this was found to be 0.07, 0.33 and 0.67 gm. respectively on the three rations and in the case of phosphorus 0.70, 4.23 and 5.41 gm. There was therefore a marked difference between the urinary excretion of calcium and phosphorus. The average daily excretion of calcium was 4 and 8 mg., respectively, on rations 2 and 3 as compared with 1 mg. on the control ration, while the daily phosphorus excretion on rations 2 and 3 rose from an average of 90 mg. on the control ration to 500 and 675 mg., respectively. While these figures give an estimate of the relative amounts of calcium and phosphorus excreted daily by the kidneys, it should be noted that the actual daily excretion probably varied considerably from the averages

because of the variations at different periods of the experiment in the intake and rate of deposition in the body.

The smaller amount of fat in the animals receiving a larger amount of the calcium and phosphorus than the controls was probably due in small part to an interference in absorption of the food material. An estimate of the amount of food that remained unabsorbed was obtained by subtracting the ash content of the feces from the total dry weight. In the case of the controls this amounted to an average of 30.7 gm. and for the animals on rations 2 and 3, 35.6 and 37.2 gm., respectively, or 4.9 and 6.5 gm. more than on the control ration. From the formulae proposed for the conversion of carbohydrate into body fat (compare Rapport, '30), it can be estimated that the food mixture that escaped absorption on rations 2 and 3 would have yielded less than 2 and 2.5 gm. fat, respectively, whereas the body fat on these two rations was 4.2 and 10.1 less than on the control ration. The major portion of the differences in the fat content of the animals on the three rations must therefore have been due to factors other than differences in absorption of foodstuff.

SUMMARY AND CONCLUSIONS

When large amounts of calcium and phosphorus were added to the rations of albino rats practically all the excess calcium was recovered in the feces, whereas the phosphorus in the feces was considerably less than the excess intake. The kidneys were therefore called upon to excrete very little more calcium but much more phosphorus than on the control ration.

A small portion of the excess of absorbed calcium but none of the phosphorus was stored in the body.

The percentage gain in body weight was not affected by the high calcium-phosphorus intake. The dry weight of the body, however, was definitely lower.

The fat content of the body was significantly reduced by the increased intake of calcium and phosphorus. The diminution in the total fat and dry weight was approximately the same.

Both the fat and dry weight of the body showed a progressive decline with a progressive increase in the calcium-phosphorus intake.

The large amount of calcium and phosphorus interfered to a slight extent with the absorption of food material as shown by combustion of the feces. The effect on absorption, however, could account only in a small degree for the diminution in the fat content of the body.

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SIMULTANEOUS APPEARANCE OF A POSITIVE LINE TEST AND RADIOACTIVE PHOSPHATE DEPOSITION IN THE RACHITIC RAT METAPHYSIS [1]

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FOUR FIGURES AND ONE PLATE (FIVE FIGURES)

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Artificially produced radioactive isotopes of several physiologically important elements are proving of great value in studies of intermediary metabolism. This is especially true of the unstable isotope of phosphorus (P^{32}) as applied to studies of the calcification processes. Due to the high degree of biological standardization which has been achieved in the technic of assay for vitamin D, the rachitic rat, as prepared for this purpose, offers a very satisfactory tool for the investigation of vitamin D action as traced by radioactive phosphorus. Within the past year several preliminary reports of investigations of this type have appeared. Dols and his associates ('37, '38 and '38 a) at Amsterdam have reported that vitamin D appears to have no characteristic mode of action on the absorption or re-excretion of administered phosphorus in the gut of the rachitic rat. Neither were they able to detect any difference between normal and rachitic rats in the rate

[1] These data are taken from a portion of the material presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of Rochester, by Marian L. Manly.

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of synthesis of phospholipid. Using rachitic chicks, they have found that the phosphorus metabolism of the bone as a whole is more intense than that of normal birds. Furthermore, the radioactive phosphorus concentrated more rapidly in the epiphyses than in the diaphyses of the same bones. Very recently, Cohn and Greenberg ('39) have reported, in abstract form, the results of experiments on rats from which they concluded: "The influence of vitamin D on the deposition of inorganic phosphorus in the bone of rachitic rats must be ascribed to a more direct effect than one merely resulting from an increased absorption. A specific effect of vitamin D on organic bone phosphorus and a specific role of this fraction in bone formation are indicated."

This paper constitutes one of a series from this laboratory employing radioactive phosphorus (P^{32}) for the investigation of problems relating to the calcification of bones and teeth.

EXPERIMENTAL

Radioactive sodium phosphate.[4] A sample of Na_2HPO_4 containing radioactive phosphorus was dissolved in distilled water and made up to a convenient volume from which aliquots were taken for further dilution to concentrations suitable for administration to rats.

Plan of procedure. Two series of young rachitic rats from our standardized vitamin D assay colony were used, the first consisting of 18, the second of 38 animals, making a total of 56. They were distributed among the experimental and control groups as shown in table 1. All animals received a single dose each of radioactive phosphorus (as Na_2HPO_4) in 1.0 cc. of water by stomach tube. In series 1, the dose of phosphorus was 0.1 mg. and yielded 96,000 c.p.m.[5] at the time administered. In series 2, 0.2 mg. of phosphorus yielded 80,000 c.p.m. Vitamin D, as crystalline D_2 dissolved in propylene glycol, was also given by stomach tube at the same time as the phos-

[4] The authors are indebted for this material to the Radiation Laboratory of the University of California.

[5] Counts per minute (c.p.m.) as determined by our scale-of-four counters.

phorus. The amount of glycol solution was 0.2 cc. per rat and for series 1 contained 10 I.U. and for series 2, 15 I.U. per dose. At the time of the experiment, the animals averaged 80 gm. in weight and 50 days of age and had been prepared by a period of 23 days on the rachitogenic diet 2965 of Steenbock and Black ('25).

Samples. At various times after dosing, as indicated in table 1, the animals were sacrificed by decapitation and the

TABLE 1

Deposition of radioactive phosphate in the rachitic rat metaphysis

TIME	NUMBER OF RATS	DOSE P AS NA_2HPO_4	RADIOACTIVITY COUNTS PER MINUTE	DOSE OF VITAMIN D	AVERAGE FRACTION TOTAL RADIOACTIVITY PER GRAM OF FRESH TISSUE			LINE TEST
					Blood	Diaphyses	Metaphyses	
hours		*mg.*		*I.U.*	%	%	%	
				Series 1				
2	4	0.1	96,000	10	0.15	1.31	0.10	
4	2	0.1	96,000	10	0.25	1.58	0.21	
8	3	0.1	96,000	10	0.29	2.26	0.33	
12	2	0.1	96,000	10	0.27	2.27	0.29	
24	3	0.1	96,000	10	0.24	3.10	0.38	
92	2	0.1	96,000	10	0.22	3.46	1.71	
92	2	0.1	96,000	None	0.17	3.10	0.57	
				Series 2				
2	3	0.2	80,000	15	0.08	0.81	0.09	—
2	1	0.2	80,000	None	0.19	2.03	0.20	—
5	3	0.2	80,000	15	0.18	1.68	0.35	—
5	2	0.2	80,000	None	0.18	1.70	0.28	—
8	3	0.2	80,000	15	0.15	1.23	0.18	—
8	2	0.2	80,000	None	0.21	2.11	0.29	—
12	3	0.2	80,000	15	0.18	1.53	0.17	—
12	2	0.2	80,000	None	0.25	2.09	0.38	—
24	3	0.2	80,000	15	0.31	2.71	0.62	—
24	2	0.2	80,000	None	0.27	1.90	0.50	—
54	3	0.2	80,000	15	0.21	2.01	0.63	±
54	2	0.2	80,000	None	0.19	2.30	0.42	—
72	2	0.2	80,000	15	0.22	3.19	2.46	+
72	2	0.2	80,000	None	0.24	2.79	0.66	—
96	3	0.2	80,000	15	0.22	2.31	2.31	++
96	2	0.2	80,000	None	0.15	2.50	0.55	—

blood collected in a flask containing 0.2 cc. of saturated sodium citrate solution and weighed. Both tibias (with fibulas attached) were dissected out and slabs 1.5 to 2.0 mm. thick were sectioned longitudinally from the center of the bones in the plane of attachment between the tibia and fibula. The slabs were then stained with silver nitrate according to the standard line test procedure and photographed. With the micro-jigsaw apparatus of Deakins and Manly ('39), a sample was cut from the metaphysis to include the area of new calcifica-

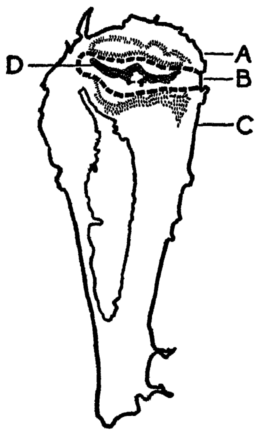

Fig. 1 Diagrammatic drawing of tibial slab. A, B and C are epiphysis, metaphysis and diaphysis, respectively. D represents the area of new calcification shown by silver nitrate staining. The metaphyseal area within the dotted line was that cut out by the micro-jigsaw for determination of radioactive phosphorus content of newly deposited bone salt.

tion (the 'line') as illustrated by the drawing in figure 1. The weight of these blocks of metaphyseal cartilage averaged 12 to 13 mg. Samples of tibial diaphysis (including the marrow) were also taken. The two samples of metaphysis and diaphysis from each rat were pooled and weighed. All samples, including blood, were dried on a hot plate, ashed in an electric oven at 500° C. over night, the ash dissolved in concentrated HNO_3 and HCl, made up to 2.0 cc. volume and the radioactivity determined with a scale-of-four Geiger-Müller counter (Bale, Haven and LeFevre, '39). The phosphorus in

the doses of Na_2HPO_4 solution was determined in duplicate by the method of Holtz ('29).

RESULTS

To allow for small differences in body weight of the rats, all of the results were multiplied by the body weight and divided by 80 gm. (the approximate average of the group). Hence, the data are expressed as per cent of the total dose of radioactive P per gram of fresh tissue per 80 gm. rat. The average values for the rats of each time period for the three tissues studied are to be found in table 1. The data are also expressed graphically in figures 2, 3 and 4.

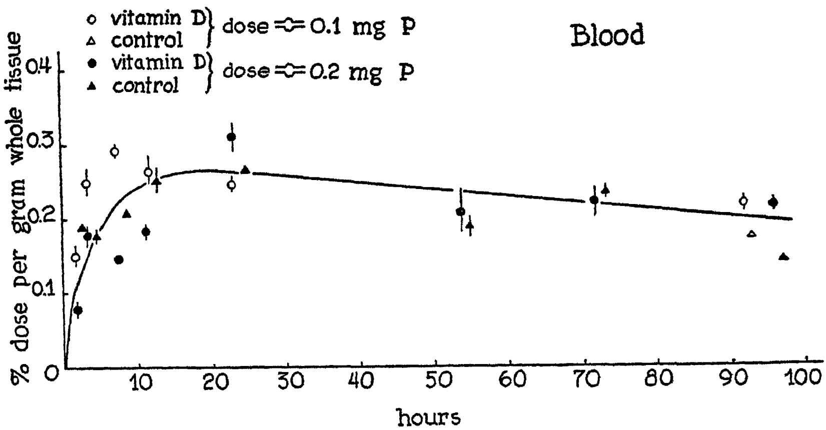

Fig. 2 Showing the entrance of radioactive phosphorus into the blood after the administration of a single dose of Na_2HPO_4 containing the P^{32} isotope. Note that vitamin D has no effect on the values found after either 0.1 or 0.2 mg. of P.

Blood. The radioactive phosphorus in the blood (fig. 2) reaches a maximum of 0.27% in less than 24 hours and decreases slowly to 0.20% in 4 days. There is no apparent difference between the amount of radioactive phosphorus in the blood of the rats fed vitamin D and that of the controls.

Diaphyses. The radioactive phosphorus in the diaphyses (fig. 3) rises slowly to 2.5% in 24 hours and continues to rise throughout the experimental period. Again, there is no apparent difference between vitamin D-fed rats and controls.

Metaphyses. The values for the vitamin D rats and the controls both fall along the same line at 0.5% up to 54 hours (fig. 4). After 54 hours, however, the values for the vitamin

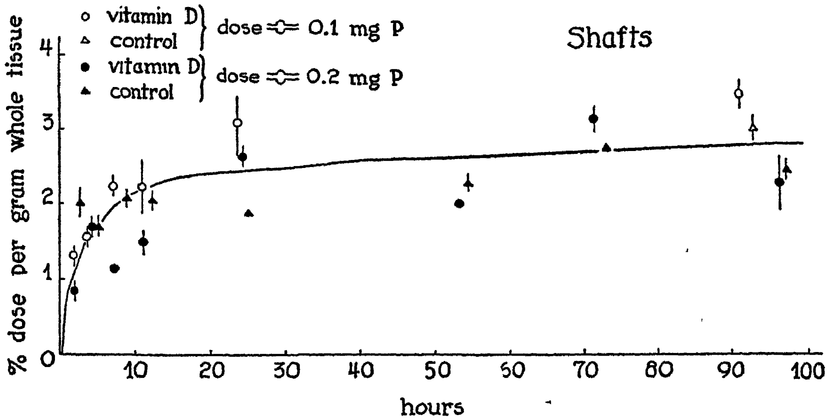

Fig. 3 Showing the entrance of radioactive phosphorus into the diaphyseal shafts. Here also, vitamin D has no effect.

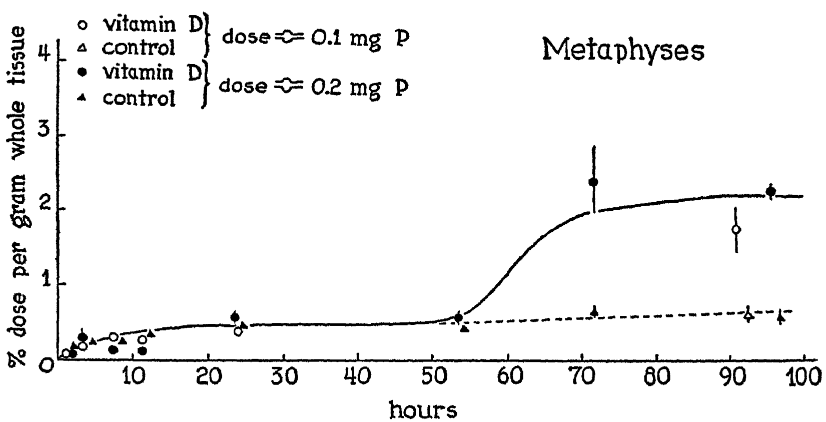

Fig. 4 Showing the retention of radioactive phosphorus in the metaphyseal cartilage. Note that at 72 hours the values in the vitamin D fed rats are significantly higher than those of the controls.

D-fed rats rise above 2.0% while the controls remain at the 0.5% level. The average degree of healing produced by vitamin D in series 2 is indicated in the last column of table 1.

These results show that the development of the 'line' of new calcification in the rachitic metaphysis occurs concomitantly with the entrance of the radioactive phosphorus into this area.

DISCUSSION

Almost nothing is known concerning the metabolic processes by means of which vitamin D mediates the formation of new bone salt in the rachitic animal. Various theories have been proposed among which that of Harris and his associates ('31 and '32) has probably been most widely cited. These workers believed that the chief function of vitamin D is to increase the "net absorption of calcium and/or phosphorus" from the gut. Nicolaysen, in a series of papers appearing in 1937 ('36 and '37 a), indicated that the influence of vitamin D is on absorption of calcium and that it has very little effect on the absorption of phosphorus. However, in the same year, Nicolaysen ('37) further showed that rachitic rats on the 2965 diet absorb about twice their daily requirement of calcium and only about 30% of their phosphorus needs. As pointed out by Morgareidge and O'Brien ('38), these findings do not support the calcium absorption theory of vitamin D action, at least for low-phosphorus rickets in the rat. That the rat is peculiar in its ability to adjust its mineral economy to a normal level when deprived of vitamin D, provided the intake of calcium and phosphorus remains within normal limits, is a fact that has been emphasized by many investigators. Nevertheless, O'Brien and Morgareidge ('39) have shown that small doses of vitamin D are very effective in increasing the efficiency with which additional phosphorus is utilized by the rachitic rat on the 2965 diet. If, therefore, the observations of Nicolaysen and others are correct that phosphorus absorption from the gut is independent of vitamin D, the conclusion that this vitamin plays an important role in the intermediary metabolism of the bone forming elements must be seriously considered. In this connection, we feel that the experiments with radioactive phosphorus reported in the present paper are significant.

In interpreting these results, it is to be emphasized that the total amount of phosphorus actually administered was only 0.1 to 0.2 mg., an amount too small to constitute a factor per se in the healing of rickets, yet, the high content of the radioactive isotope in the sample (80,000 c.p.m. per dose) made the tracing of the tagged phosphorus relatively easy.

Of major interest is the finding that vitamin D had no effect on the entrance of phosphorus into the blood or into the tibial diaphysis. The values found are in general agreement with those previously reported from this laboratory (Manly and Bale, '39). In the rachitic metaphyses, however, the effect of vitamin D is clearly seen. The entrance of phosphorus into this structure corresponds almost exactly with the appearance of the 'line' produced by silver nitrate staining in those animals which had received a single dose of the vitamin some 54 or more hours previously. In the metaphyses of the control animals the 'line' did not appear nor did the content of radioactive phosphorus increase. We feel that such evidence lends definite support to the conclusion that vitamin D has for at least one of its actions, a direct influence on the metabolic factors controlling calcification. This is in contradistinction to any possible role it may play in intestinal absorption.

The fact that from 54 to 72 hours must elapse between administering the vitamin and the entrance of phosphorus into the fixed bone salt which is responsible for the line test may be taken as additional evidence that mere absorption is not the limiting factor. Kramer, Shear and Siegel ('31) showed that simple addition of phosphorus to the 2965 diet may result in the inception of healing in rachitic rats in as short a time as 12 hours. The inference is, therefore, that under the conditions described here, the time interval between administration of the vitamin and the beginning of new calcification is that required for the metabolic action of vitamin D to mobilize the phosphorus required for the healing process.

SUMMARY

Following a single dose of Na_2HPO_4 containing the radioactive isotope (P^{32}) accompanied by a single dose of vitamin D_2, determinations have been made of the radioactive phosphorus content of whole blood and of the tibial diaphysis and metaphysis of rachitic rats. The data show that vitamin D has no influence on the entrance of phosphorus into the blood or into the diaphyseal portion of the tibia. In the metaphysis, however, the healing produced by the vitamin (as shown by the appearance of a positive line test) occurs concomitantly with a significant increase in the content of radioactive phosphorus. These effects in the metaphysis are seen only after the elapse of 54 to 72 hours subsequent to the administration of the vitamin. It is pointed out that the results support the conclusion that the mode of action of vitamin D is not limited to the control of intestinal absorption of the elements concerned in calcification.

ACKNOWLEDGMENTS

We are indebted to the Wisconsin Alumni Research Foundation and to the Rockefeller Foundation for partial financial support of this work. We wish to acknowledge the assistance of Dr. William F. Bale for the construction and maintenance of the Geiger-Müller counters and of Miss Sylvia Levy in making many of the radioactivity determinations. Dr. Brian O'Brien and Dr. Martin Deakins kindly loaned the photographic equipment used in recording the line tests.

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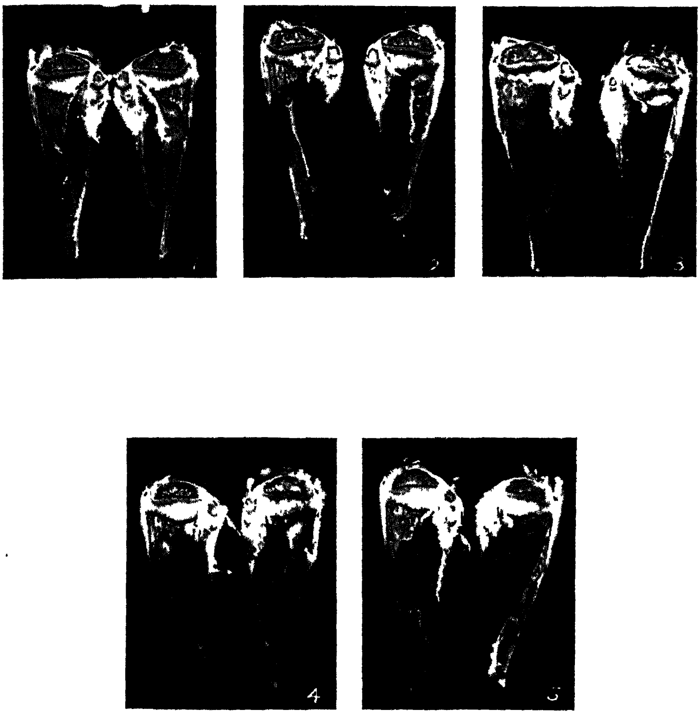
PLATE 1

EXPLANATION OF FIGURES

1, 2, and 3 Left and right tibial slabs stained with silver nitrate 96 hours after the administration of 15 I.U. of vitamin D_2 and 0.2 mg. of phosphorus (as Na_2HPO_4) containing radioactive P^{32} corresponding to a total of 80,000 c.p.m. Per cent of total radioactivity per gram of fresh metaphyseal cartilage was 1.2, 2.6 and 3.1, respectively, for the three rats. The intensity of the line of new calcification increases with increasing content of the radioactive isotope.

4 and 5 Tibial slabs from two 96-hour control rats which received the same dose of phosphorus but no vitamin D. Note absence of healing. Per cent of total dose of radioactivity per gram of metaphyseal cartilage in these two rats was 0.75 and 0.35, respectively.



THE EFFECT OF HEAT AND SOLVENTS ON THE NUTRITIVE VALUE OF SOYBEAN PROTEIN [1]

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Reviewing the literature on the effect of heat on the nutritive value of proteins, Morris ('37) made the statement that "in view of the mass of contradictory evidence further confirmation of the work of Hayward seems essential." Morris was referring to the observations made on meat protein (Morgan and Kern, '34), casein (Greaves and Morgan, '34; Block, Jones and Gersdorff, '34), and skim milk powders (Fairbanks and Mitchell, '35) confirmed by other studies which revealed that the nutritive value of the proteins had been lowered by heat. This was in contrast to the demonstration of Hayward, Steenbock and Bohstedt ('36 a) that the proteins of expeller and hydraulic soybean oil meals prepared at high temperatures were nutritionally superior to those prepared at low temperatures. Later Wilgus, Norris and Heuser ('36) working with chicks at Cornell reported that solvent soybean oil meal was superior to the high temperature expeller and hydraulic soybean oil meals. They suggested that since the temperature used in the solvent process was considerably lower than that used in the preparation of the expeller and hydraulic meals, a factor other than temperature was responsible for the difference.

The question arose as to whether treatment with certain fat solvents could alter the protein molecule of the soybean

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

so as to make it more available inasmuch as results obtained by Mashino ('29) and Kajizuka ('35) had suggested such a possibility.

Other investigators have given consideration to the removal of hypothetical toxic factors by solvents or their destruction by heat. Osborne and Mendel ('17), who studied the nutritive value of soybeans as early as 1917, stated that "evidently there is nothing toxic in the raw meal, for none of the rats which ate it died." Waterman and Johns ('21) reported that the increased digestion of phaseolin after heating was due to the "loss of a protein toxicity or the destruction of an associated toxic substance." Shrewsbury and co-workers ('32) observed that cooking increased the digestibility of the soybeans only slightly. Their theory was that heating caused the removal or destruction of certain materials of a toxic nature.

Since Hayward, Steenbock and Bohstedt ('36 a and b) had shown that heated soybeans had a higher biological value than raw soybeans and that cystine additions to raw soybeans increased the amount of nitrogen retained, it appeared desirable to obtain data on sulfur balances as well.

EXPERIMENTAL

The soybeans used were of the Illini variety harvested in 1934. From these special expeller soybean meals were prepared in commercial plants and autoclaved and solvent treated samples were prepared in the laboratory. The expeller soybean meal used for our feeding trials had been exposed to the comparatively high temperature of 150°C. This was chosen in preference to others prepared at a lower temperature since soybean meal processed at such a high temperature had been found to have the highest biological value (Hayward, Steenbock and Bohstedt, '36 a). The autoclaved soybean preparations were prepared as described by Hayward, Steenbock and Bohstedt ('36 a) by autoclaving them for $1\frac{1}{2}$ hours at 17 pounds pressure.

The solvents used for the extraction of the soybeans were commercial petroleum ether, hexane and a mixture of ninety parts of hexane and ten parts of methanol. The solvent treated soybeans were prepared as follows: ground soybean and solvent were poured alternately into a percolator and then stirred. Small amounts of each were added until 2 kg. of soybeans had been used. The extraction was continued over night at room temperature. The solvent was then drained off slowly in 3 to 4 hours. Four extractions were made. For the first two extractions 2 to $2\frac{1}{2}$ liters of solvent were used; for the later extractions 1 to $1\frac{1}{2}$ liters.

The soybean residue was freed from most of the solvent in 2 hours with frequent stirring before an electric fan. Finally it was exposed in shallow trays to the air over night. The solvent was removed from the oil at room temperature with nitrogen under reduced pressure.

The soybean residues and oils were fed in the following combinations: a) residue plus oil prepared at room temperature, and b) residue and oil autoclaved together at 17 pounds pressure for $1\frac{1}{2}$ hours. The composition of the rations in which these were fed was as follows: solvent treated soybeans 46 parts, cod liver oil 2 parts, salt mixture (Steenbock and Nelson, '23) 4 parts and cooked starch 48 parts. The raw or autoclaved soybeans were fed in the following ration: soybeans 45, cod liver oil 2, salt mixture 4 and cooked starch 49. In the soybean oil meal ration thirty-eight parts of the meal were mixed with eight parts of soybean oil so as to equal the ratio in which they occur in the raw soybean. The protein was fed at a level of 18%. To insure that most of the sulfur came from the soybeans, sulfates were omitted from the salt mixture.

Routine care of animals

The daily food allowances, weighed to centigrams, were fed to the rats at approximately the same time of day. The maximum level of equalized food intake was determined by a preliminary feeding period of 3 to 5 days. During the experi-

mental periods of 4 to 7 days all rats in a group consumed the same amount of food. They were weighed at the beginning and end of each preliminary and experimental period.

Description of metabolism cages and care of excreta

The rats were quartered in individual metabolism cages of glass cylinders, 9 inches in diameter, resting on screens over glass funnels, 10 inches in diameter and 14 inches high. The separation of the feces from the urine was accomplished by allowing the feces to collect in an antiseptic chamber containing blotting paper soaked in formaldehyde as the urine flowed over a glass bulb through a small funnel to a collecting flask containing toluene. The receivers were emptied daily into storage flasks and rinsed with distilled water. In the experiments for the determination of sulfur distribution in the urine, the filtered urine preserved with toluene was stored at 5°C. In groups 1 and 2 sulfur was determined on the urine of each rat for each period (table 2). In the other groups it was determined on composite samples from four rats for each period (table 1).

To prevent loss of sulfur the feces were treated with 10 cc. of 2% copper acetate (Cuthbertson and Turnbull, '34) in 50 cc. Erlenmeyer flasks. They were then dried first on a sand bath at 80 to 100°C. and then in an oven at 100°C. until constant in weight. Analyses for sulfur were made on ground samples composited the same as the urine samples.

When nitrogen was determined on the urine and the feces, 10 cc. of 2% H_3PO_4 were added to the storage flasks. Analyses for nitrogen were made on separate composites of urine and of feces from the four rats in the group for each period.

Methods of analyses

Total sulfur of the rations and feces was determined by sodium peroxide fusion. The fat of the ration was extracted with anhydrous ether before analysis to prevent foaming during fusion. This was permissible because analysis of soy-

TABLE 1

Sulfur absorbed and retained on heated versus raw soybean diets

GROUP [1]	PERIOD [2]	WEIGHT OF RATS AVERAGE	FOOD CONSUMED	SULFUR [6] In ration Per cent	In ration Weight	Excreted Feces	Excreted Urine	Absorbed Weight	Absorbed Per cent	Retained Weight	Retained Per cent
				Soybean oil meal (expeller—150°C.)							
		gm.	gm.	%	gm.	gm.	gm.	gm.	%	gm.	%
1	1	97	96	0.165	0.159	0.060	0.058	0.099	62.3	0.041	41.4
2	2	111	86	0.160	0.137	0.048	0.062	0.089	64.2	0.027	30.3
				Whole soybean, raw							
1	2	96	96	0.164	0.157	0.061	0.078	0.096	61.1	0.018	18.7
2	1	110	93	0.167	0.155	0.063	0.081	0.092	59.4	0.011	12.0
				Whole soybean, autoclaved [3]							
3	1	128	200	0.168	0.336	0.129	0.116	0.207	61.6	0.091	44.0
4	1	124	207	0.168	0.348	0.131	0.120	0.217	62.4	0.097	44.7
				Whole soybean, raw							
3	2	142	202	0.168	0.339	0.118	0.193	0.221	65.2	0.028	12.7
4	2	141	215	0.168	0.361	0.123	0.183	0.238	65.9	0.055	23.1
				Ether extracted soybean plus oil							
5	1 [4]	117	217 (auto)	0.175	0.379	0.146	0.120	0.233	61.5	0.113	48.5
5	2 [5]	124	211 (raw)	0.177	0.373	0.143	0.164	0.230	61.7	0.066	28.7
				Hexane extracted soybean plus oil							
6	1 [4]	97	173 (auto)	0.174	0.301	0.107	0.101	0.194	64.5	0.093	47.9
6	2 [5]	101	161 (raw)	0.166	0.267	0.102	0.137	0.165	61.8	0.028	17.0
				Hexane-methanol extracted soybean plus oil							
7	1 [4]	93	173 (auto)	0.178	0.308	0.108	0.092	0.200	64.9	0.108	54.0
7	2 [5]	95	151 (raw)	0.172	0.261	0.090	0.144	0.171	65.5	0.027	15.8

[1] Each group had four male rats, ages ranging from 5 to 7 weeks.
[2] Period consisted of 4 days for groups 1 and 2; of 7 days for groups 3 to 7 inclusive.
[3] Soybeans were autoclaved at 17 pounds pressure for 1½ hours.
[4] Extracted soybean residue and oil autoclaved together 1½ hours at 17 pounds pressure.
[5] Extracted soybean residue and oil prepared at room temperature.
[6] In groups 1 and 2 sulfur was determined on the urine of each rat for each period (table 2). In the other groups sulfur determinations were made on composite samples of urine from four rats for each period. Analyses for sulfur of the feces were made on composite ground samples from the four rats in a group for each period. All figures represent the composites of four rats in a group for each period.

bean oil revealed the presence of only traces of sulfur, viz., 0.01%.

Total sulfur of the urine was determined by the Benedict-Denis method ('09, '10); and combined inorganic and ethereal sulfur by Folin's methods (Folin, '05, '06). Ethereal and neutral sulfur were obtained by difference.

Results

Table 1 contains the results of the sulfur balances of the heated and raw soybeans and of the soybeans treated with the various solvents. The sulfur was found to have been absorbed to the same degree on all the rations, with an average coefficient of digestibility of 63.0%. The retention of sulfur, on the contrary, varied greatly. The amount retained on the heated soybean rations, viz., commercial soybean meal and soybeans autoclaved in the laboratory ranged from 30.3 to 44.7% of the intake, the average being 40.1%; on the raw soybean rations, 12.0 to 23.1% with an average of 16.6%.

With the solvent-treated soybean prepared at room temperature, more sulfur was retained from rations in which the residues and oil from these had been autoclaved together than from rations in which they were fed together with no heat treatment. In the former the sulfur retention averaged 50.1%, in the latter 20.5. Apparently the solvent itself had no beneficial effect.

In table 2 are given the amounts of total sulfur excreted in the urine by individual rats for periods of 4 days. All rats eliminated less sulfur on the heated than on the raw soybean ration. Collectively, groups 1 and 2 of four animals each on raw soybeans excreted 34.5 and 30.6% more sulfur in the urine than those receiving the heated soybeans. For groups 3 to 7 inclusive the same relations are seen; a higher percentage of sulfur was excreted in the urine on the unheated than on the autoclaved soybeans (table 1). In table 3 are given the results on nitrogen balances. These show that on heated soybeans the rats retained about 2.5 times as much sulfur and about 1.8 times as much nitrogen as on the raw soybeans.

TABLE 2

Urinary sulfur of individual rats in 4-day periods

GROUP [1]	RAT	WEIGHT OF RATS	PERIOD OF 4 DAYS	SOYBEAN OIL MEAL (EXPELLER—150°C.)		PERIOD [2] OF 4 DAYS	WHOLE SOYBEAN, RAW	
				Food consumed	Sulfur in urine		Food consumed	Sulfur in urine
	no.	*gm.*	*no.*	*gm.*	*gm.*	*no.*	*gm.*	*gm.*
1	1	96	1	24	0.016	2	24	0.021
	2	97		24	0.013		24	0.018
	3	100		24	0.015		24	0.020
	4	95		24	0.014		24	0.019
Total				96	0.058		96	0.078
2	5	114	2	24	0.016	1	24	0.020
	6	102		19	0.013		22.5	0.020
	7	112		24	0.019		24	0.023
	8	112		19	0.014		22.5	0.018
Total				86	0.062		93	0.081

[1] In groups 1 and 2 sulfur was determined on the urine of each rat for each period of 4 days.

[2] The sequence of periods 1 and 2 was reversed for group 2.

TABLE 3

Sulfur and nitrogen absorbed and retained on heated versus raw soybean diets

Group 2

DIET	FOOD CONSUMED	IN RATION		EXCRETED		ABSORBED		RETAINED	
		Per cent	Weight	Feces	Urine	Weight	Per cent	Weight	Per cent
Sulfur [1]									
	gm.	*%*	*gm.*	*gm.*	*gm.*	*gm.*	*%*	*gm.*	*%*
Soybean oil meal (expeller—150°C.)	86	0.160	0.137	0.048	0.062	0.089	64.2	0.027	30.3
Whole soybean raw	93	0.167	0.155	0.063	0.081	0.092	59.4	0.011	12.0
Nitrogen [1]									
Soybean oil meal (expeller—150°C.)	86	2.57	2.20	0.455	1.172	1.745	79.3	0.573	32.8
Whole soybean raw	93	2.86	2.66	0.661	1.645	1.999	75.1	0.354	17.7

[1] All figures represent the composites of four rats in a group for a period of 4 days.

Since more urinary sulfur was excreted on the raw soybean ration than on the heated, the distribution of sulfur in the urine was of interest. If the sulfur-containing fraction of the protein of the raw soybean was not available because of its conjugation with some toxic factor, then it was possible that an increase in the neutral sulfur would have occurred with a corresponding decrease in the ethereal and inorganic sulfur forms. Four male rats 12 weeks of age and averaging 212 gm. in weight were used to determine this.

TABLE 4

Distribution of urinary sulfur on diets containing heated and raw soybeans

GROUP [1]	PERIOD [2]	WEIGHT OF RATS	FOOD CONSUMED	SULFUR [3]									
				In ration		Urine					Relation to total		
				Per cent	Weight	Total	Inorganic	Inorganic and ethereal	Ethereal	Neutral	Inorganic	Ethereal	Neutral
Whole soybean, raw													
		gm.	*gm.*	%	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	%	%	%
8	1	224	126	0.167	0.210	0.159	0.103	0.119	0.016	0.040	64.8	10.1	25.1
Soybean oil meal (expeller—150°C.)													
8	2	212	129	0.160	0.206	0.133	0.082	0.097	0.015	0.036	62.3	11.3	26.4

[1] Group 8 had four male rats approximately 12 weeks of age.

[2] Period consisted of 5 days.

[3] All figures represent the composites of four rats in a group for each period.

The composition of the rations, the routine care of the animals, the metabolism cages and methods of analyses have already been described. Table 4 shows the inorganic, ethereal and neutral sulfur excreted in relation to the total. The results of the sulfur balance studies of these adult rats are not presented since the coefficients of digestibility on the heated and raw soybeans were similar to those of the young animals.

DISCUSSION

Some workers (Mitchell and Smuts, '32; Shrewsbury and Bratzler, '33) have contended that soybeans are deficient in cystine. They based their conclusion upon the observation

that ether extracted soybeans plus cystine produced a greater increase in the weight of animals than unsupplemented soybeans. Both the ether extracted soybeans and the unsupplemented soybeans were fed unheated.

Csonka and Jones ('34), on the other hand, stated that "The analytical data do not support the idea of a quantitative deficiency of cystine in soybean proteins, but they do admit the possibility of a qualitative deficiency due to low availability of amino acids when the raw meal is fed, particularly when soybeans containing the lower cystine values are used."

Osborne and Mendel ('17) pointed out that heat improved the nutritive value of the soybean. Extracting the soybean with ether failed to increase its biological value. Cooking the soybean made it more palatable than the raw. In this manner the increased food consumption resulted in a better gain. Vestal and Shrewsbury ('32) concluded from metabolism studies that improved growth on cooked or roasted soybeans was due to an increase in food consumption and a slight increase in the digestibility of nitrogen.

Hayward, Steenbock and Bohstedt ('36 a) found that cystine supplemented the raw soybean but failed to cause any improvement with the heated soybean. From both growth studies and nitrogen balance experiments it was concluded that heat was the factor which caused the protein to be well utilized by the animal.

In the present study the observation that heat improved the nutritive value of the soybean was confirmed. Sulfur metabolism studies showed clearly that the digestibility of the protein was approximately the same for the raw and heated soybean. The difference lay in the availability of the products of protein digestion. Less urinary sulfur was excreted on the heated soybean than on the raw, therefore more was retained.

The results on the soybeans treated with various solvents are contrary to those of others (Wilgus, Norris and Heuser, '36; Mashino, '29; Kajizuka, '35). These investigators be-

lieved that the action of the solvent itself was the factor which increased the biological value of the protein. However, the Cornell workers used commercial solvent soybean oil meal which had been exposed to a temperature of 98°C. for 15 minutes and to temperatures of 45 to 78°C. for an unspecified period. The Japanese workers did not report their temperatures. In our preparations the soybeans were extracted at room temperature, approximately 20 to 25°C., and the solvent was removed from the oil at the same temperature. Therefore, heat can be excluded as a determining factor in our laboratory preparations. Heat was applied only after the oil had been extracted from the soybean to permit comparing the effect of solvents alone and of solvents plus heat.

The percentage distribution of urinary sulfur, with respect to the inorganic, ethereal and neutral forms, was the same for heated and raw soybeans. However, in comparing the actual amounts of the different forms of sulfur excreted on the two diets, it is seen that a decrease in the excretion of total, inorganic, ethereal and neutral sulfur occurred on changing to the heated diet.

SUMMARY

Sulfur and nitrogen balances on rats confirmed previous findings that the nutritive value of soybean protein is improved by heat. The claim that treatment with certain solvents such as hexane and methanol have a similar effect was not substantiated. It appears that soybeans contain a S and N containing complex which is absorbable but cannot be used for tissue building purposes. Heating the soybeans makes it available.

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THE SYNTHESIS OF FAT FROM PROTEIN BY THE ALBINO RAT

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Whether protein may be transformed into fat by higher animals has long been a question of particular interest to students of nutrition. The generally accepted view has been that such a transformation may take place. Protein and certain amino acids yield considerable proportions of dextrose when fed to diabetics or to animals which have been depancreatized or treated with phlorizin. Since dextrose from other sources is readily changed into fat in the animal body, it is reasonable to expect that dextrose derived from protein also may be so transformed. However, Longenecker ('39) appears to have been the first to present direct experimental evidence that normal higher animals can synthesize fat from protein. In his experiments, male albino rats were grown on an adequate stock diet to weights of about 260 gm. They were next fasted until they had lost 27 to 28% in weight. The carcasses of some, after rejection of certain portions, were then analyzed for fat content. The remaining rats were transferred to a high-protein ration until they had practically recovered their former weights, at which time their carcasses were similarly analyzed for fat content. Longenecker reported that more than one-third of the gain in weight of the second group was due to the deposition of fat, a quantity representing twenty to thirty times the amount of lipids contained in the total ration consumed during the refeeding period.

The present writers had completed the experiments herein described and had practically completed their descriptive manuscript before Longenecker's paper appeared. Though their results now become merely corroborative, nevertheless the data are of interest because the problem was attacked by a method which differed in some details from that employed by Longenecker. The purpose of the present experiments was to determine whether young albino rats could materially increase their store of fat when fed a diet consisting chiefly of casein and practically free from fat and carbohydrates.

PREPARATION OF DIET

The fat- and carbohydrate-free diet contained purified casein, 94%; yeast vitamins, 2%; salt mixture, 4%; plus vitamins A and D equivalent to 2% of cod liver oil, added in the form of an ether extract of the saponified oil. The ingredients were prepared as follows:

Commercial casein was extracted with ethyl ether by percolation until practically free from ether extract and was then treated with successive portions of boiling 95% alcohol on a steam bath until practically free from alcohol-soluble material. Analysis of the purified casein by digestion with 30% potassium hydroxide solution, acidification, and extraction with petroleum ether showed practically no fatty acids. Water-soluble vitamins were supplied in the form of commercial concentrate from brewers' yeast, which yielded only a trace of fatty acids when analyzed by the above method. Reducing sugar was not found in either the casein or the yeast vitamins. The salt mixture was that regularly employed in feeding experiments with rats in this laboratory.

DETERMINATION OF TOTAL FATTY ACIDS IN RATS

Each entire carcass was frozen hard at —9°C., chopped into pieces, and passed several times through a meat grinder. Approximately 10 gm. of the ground material was weighed into

a 150-cc. beaker, 40 cc. of 30% potassium hydroxide solution was added, and the mixture was heated on a steam bath until the soft tissues were dissolved. Ethyl alcohol was added and heating was continued until saponification was completed. The material was transferred to a separatory funnel and acidified with hydrochloric acid. When cool, the mixture was extracted with two successive 100 cc. portions of petroleum ether. The ether extract was washed with successive portions of water until free from hydrochloric acid, transferred to an Erlenmeyer flask and the ether was expelled on a steam bath. The fatty acids were dried to constant weight at 100°C.

FEEDING EXPERIMENTS

Two closely similar male albino rats were selected from each of seven litters. One rat of each litter pair was chloroformed at the beginning of the experiment, frozen, ground, and analyzed for total fatty acid, with inclusion of intestinal contents. The other rat of each pair was fed the fat- and carbohydrate-free diet for 60 days and was then likewise chloroformed, frozen and analyzed for total fatty acid. During the feeding period each rat was kept in an individual cage with a raised screen bottom, and was supplied with feed in a self-feeder, as well as with water. The rats were weighed twice weekly and the quantity of feed eaten was recorded. The results of this experiment are shown in table 1 and for comparison the results obtained with a group of rats fed a diet containing 5% of lard are included.

All rats fed the fat- and carbohydrate-free diet developed pronounced symptoms of linoleic acid deficiency before the end of the experiment, the first symptom appearing after about 30 days. All these rats grew very slowly and appeared poorly nourished. Table 1 shows that the rats fed the fat- and carbohydrate-free diet made an average daily gain in weight of only approximately 1 gm. as compared with a gain of 3.8 gm. by the rats fed the diet containing 5% lard. The latter group of rats also utilized their feed much more efficiently.

TABLE 1

Growth in 60 days of male rats fed a fat- and carbohydrate-free diet compared with growth of male rats fed a diet containing lard and dextrose

DESCRIPTION OF DIET		RAT NO.	LITTER NO.	INITIAL WEIGHT	GAIN IN WEIGHT	FEED CON-SUMED	ENERGY INTAKE	GAIN IN WEIGHT PER 100 CAL.
Ingredient	%			*gm.*	*gm.*	*gm.*	*Cal.*	*gm.*
Casein	94	2	60C	45	89	443	1595	5.58
Yeast vitamins	2	4	64B	39	47	360	1296	3.63
Salt mixture	4	6	66A	45	64	368	1325	4.83
Vitamins A, D	Added	8	67B	40	40	352	1267	3.16
		10	67D	40	83	476	1714	4.84
		12	70A	44	73	447	1609	4.54
		14	70C	41	73	455	1638	4.46
Average				42	67	414	1493	4.43
Refined lard	5.0	1359A	59C	39	171	679	2444	7.00
Casein	20.0	1359B	61B	40	258	864	3110	8.30
Yeast vitamins	2.0	1359C	62A	40	247	833	2999	8.24
Salt mixture	4.3	1359D	62D	39	251	870	3132	8.01
Dextrose	68.7	1359E	63C	43	210	769	2768	7.59
Vitamins A, D	Added	1359F	64C	40	229	838	3017	7.59
		1359G	65D	38	231	839	3020	7.65
		1359H	66B	38	233	790	2844	8.19
Average				40	229	810	2917	7.82

STORAGE OF FATTY ACIDS BY RATS FED A FAT- AND CARBOHYDRATE-FREE DIET

In table 2 is shown the total quantity of fatty acids in the carcass of each rat at the end of the 60-day experiment in comparison with the quantity present in its litter mate at the beginning of the experiment, correlation being made for differences in the initial weights of the rats.

The results of these experiments show that each of seven male albino rats fed a fat- and carbohydrate-free diet for 60 days contained much more fatty acid at the end of the experiment than did its litter mate at the beginning. The increase ranged from 38 to 358% with an average of 257%, and the total increase for the seven rats amounted to 47.92 gm. of fatty acid. More than one-tenth of the gain in weight was constituted by fatty acids thus derived, which represents a considerably larger proportion of lipids. These figures do

not include fatty acids excreted in the feces or those oxidized by the rats. Hence, the above noted increases in fatty acid represent minimum quantities synthesized. In view of the severe conditions under which these experiments were conducted, particularly the lack of linoleic acid in the diet, the results show conclusively that the albino rat can synthesize fat from protein.

TABLE 2

Change in fatty-acid content of male albino rats when fed a fat- and carbohydrate-free diet

			AT BEGINNING OF EXPERIMENT			AT END OF 60-DAY EXPERIMENT			
RAT NO.	LITTER NO.	AGE	Initial weight	Total fatty acids: Determined	Total fatty acids: Estimated	Gain in weight	Total fatty acids	Gain in fatty acids	
		days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*%*
1	60C	21	42	2.62					
2	60C	21	45		2.81	89	11.72	8.91	317
3	64B	20	38	2.53					
4	64B	20	39		2.60	47	9.65	7.05	271
5	66A	21	45	2.61					
6	66A	21	45		2.61	64	11.66	9.05	347
7	67B	21	38	2.96					
8	67B	21	40		3.12	40	4.29	1.17	38
9	67D	21	38	3.40					
10	67D	21	40		3.58	83	8.25	4.67	130
11	70A	25	42	2.86					
12	70A	25	44		2.99	73	13.11	10.12	338
13	70C	22	41	1.94					
14	70C	22	41		1.94	73	8.89	6.95	358
	Average				2.81	67	9.65	6.85	257
	Total				19.65		67.57	47.92	

SUMMARY AND CONCLUSIONS

Young male albino rats were fed a diet consisting chiefly of casein, but free from fat and carbohydrates, for 60 days in order to determine whether the rat can synthesize fat from protein. Two similar rats were selected from each of seven litters. One rat of each pair was analyzed for total fatty acid at the beginning of the experiment; its litter mate was analyzed at the end. Each rat fed the test diet for 60 days was

found to contain a significantly larger quantity of fatty acid than had been present in its litter mate at the beginning of the experiment. The increases in fatty acid ranged from 38 to 358%, with an average of 257%. The total quantity of fatty acid in the seven rats at the end of the experiment was greater by 47.92 gm. than the total quantity in the seven litter mates at the beginning of the experiment.

The results of these experiments show conclusively that the albino rat can synthesize fat from protein, in corroboration of Longenecker's report.

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THE INFLUENCE OF A CARCINOGENIC COMPOUND ON THE HEPATIC STORAGE OF VITAMINS

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INTRODUCTION

The discovery made by Cook and his associates ('32) that certain anthracene derivatives possess the property of producing malignant tumors in animals has given rise to interesting work on the biological and chemical changes brought about by their presence in the tissues. The action of a typical carcinogenic compound, 1,2,5,6-dibenzanthracene, on mitochondria has been described by Goerner ('37–'38). Its effect on the lipid composition of these cytoplasmic structures led to a study of its influence both on the hepatic storage of the fat-soluble vitamins A and D and on the related processes of growth and calcification. In contrast with any effect that dibenzanthracene might exert on the hepatic storage of these two vitamins, its action on a third compound possessing no lipid properties was determined, namely, vitamin C.

METHODS

The two types of animals selected for use in the experiments were divided into three groups each made up of thirty-six rats and eighteen rabbits. The treated group was injected intraperitoneally with 0.1% colloidal 1,2,5,6-dibenzanthracene in 1% gelatin solution. Each rat received 3 mg. and each rabbit 10 mg. of the hydrocarbon per week. The control group of animals was similarly injected with 1% gelatin solution alone, and the normal group remained untreated. The gelatin

solutions were warmed before injection to liquefy them. The initial weights of the rats ranged from 70 to 100 gm., and of the rabbits from 1000 to 1350 gm. All were fed a commercial chow that insured normal growth and maintenance of health of each type of animal.

At various intervals animals of each group were sacrificed and the determination of vitamins in the livers immediately begun. Vitamin A was extracted according to the procedure described by Moore ('30). The ether extract obtained was then evaporated, the fatty residue dissolved in chloroform and the vitamin A determined colorimetrically with the use of a saturated solution of antimony trichloride in chloroform according to the method devised by Rosenthal and Erdélyi ('33), and modified by Andersen and Levine ('35). A biologically standardized preparation of vitamin A containing 55,000 U.S.P. units per gram was used as a standard for comparison after suitable dilution with chloroform.

To determine the effect of the hydrocarbon on the storage of vitamin C, a part of the liver from each of the above animals was set aside for its estimation as described by Birch, Harris and Ray ('33). Instead of trichloracetic acid to extract the vitamin from the liver tissues, a 2% solution of metaphosphoric acid was employed as suggested by Fujita and Iwatake ('35) to prevent deterioration of the vitamin. The acid extract was then titrated against a solution of 2,6-dichlorophenolindophenol which had been previously standardized against ascorbic acid.

The determination of vitamin D in the liver was carried out by means of biological assay described in the United States Pharmacopoeia XI ('35). Young rats of approximately the same age and weighing from 35 to 45 gm. were placed on the standard rachitogenic diet for a period of 3 weeks. During this time extracts were prepared from the livers of both rats and rabbits which had been treated with various amounts of dibenzanthracene as described above. Extracts were also prepared from the livers of the control animals which had received injections of 1% gelatin alone and of the normal

untreated animals. All extracts were made in accordance with the procedure given by Heyman ('37). The fatty residues from weighed portions of liver obtained by evaporation of the ether extracts were then made up into various dilutions with olive oil. These liver assay oils containing different concentrations of the fatty residue were fed in 0.1 cc. portions daily for 8 days to the weighed rachitic rats. Vitamin D in the form of a biologically standardized preparation was given to another group of rachitic animals for the same period, these serving as the standard reference group. A third group of rachitic rats received 0.1 cc. of plain olive oil for the same period. These gave evidence of severe rickets at the end of the test and served to show the absence of vitamin D in the olive oil used for dilutions of the fatty residues of liver extracts mentioned above. A fourth group of rachitic rats was kept throughout the assay period on the rachitogenic diet and received no other treatment. The severe rickets which these animals showed at the end of the test period indicated the persistence of the rachitic process in the untreated rats. All animals were kept in the dark during the preparation and assay periods.

From the daily dose of the biologically standardized preparation of vitamin D which was just sufficient to cause definite healing of rickets as shown by the 'line test,' the vitamin D content of the dilution of liver assay oil necessary to bring about a similar evidence of healing may be calculated. From this result the concentration of vitamin D per 100 gm. whole liver may be obtained.

In order to study the effect of dibenzanthracene on growth and calcification, a group of young rats weighing from 45 to 50 gm. was injected intraperitoneally each week for 3 months with 3 mg. of the hydrocarbon. A control group received injections of 1% gelatin and a third or normal group remained untreated. The thirty animals of each group were weighed daily at first and then twice a week during the remainer of the experiment. Each week an animal of each group was sacrificed and the hepatic vitamin A determined as described

above. The effect of the hydrocarbon on calcification was also studied on these animals by analysis of the leg bones according to the procedure described by Chick, Korenchevsky and Roscoe ('26). All animals were fed a standard commercial chow.

RESULTS

The livers of both rats and rabbits treated with dibenzanthracene were greatly enlarged, weighing from two to three times that of the normal animals. All showed macroscopic signs of marked fatty changes in the organ as first reported by Polson ('36). This, as well as the microscopic evidence of fatty degeneration and necrosis, indicated the severity of tissue damage brought about by the hydrocarbon. One might expect to find, under these circumstances, that the hepatic storage of both fat-soluble vitamins would be affected. As the results in table 1 show, the storage of vitamin A was decreased to a great extent by dibenzanthracene while that of vitamin D remained unchanged. Extraction of the alkalinized urine of the treated animals failed to show the presence of any vitamin A. The vitamin C content of the treated rats and rabbits was entirely comparable to that of the respective normal animals.

Results of the experiments on the influence of dibenzanthracene on the growth of young rats showed that the gain in weight of the treated group at the end of 1 month was only 70% that of the normal; at the end of 2 months, 60.7%; and at the end of 3 months, it had fallen to as low as 48.4%. Analysis of the liver tissues made each week showed a steadily decreasing vitamin A content in the treated group as compared with that of the normal rats. Inflammatory processes of the conjunctiva indicative of xerophthalmia were also observed in the treated rats during the latter part of the experiment. At the end of 3.5 months all the treated animals that had not been used for analytical purposes had died, while only one death occurred in the normal group. The increasing inhibition of growth caused by the injections of dibenzan-

thracene was parallelled by the progressive decrease of hepatic vitamin A resulting from damage to the liver tissues by the hydrocarbon. The effect on the storage of this vitamin was especially noticeable in those animals which had received a large total dose of dibenzanthracene. In such instances, vitamin A was entirely absent from the liver tissues, even though the content of vitamins C and D was normal.

The young rats used in the growth experiment served also to determine the influence of the hydrocarbon on calcification of osseous tissues. The results of these tests showed that the

TABLE 1

Effect of dibenzanthracene on storage of vitamins A, C and D

ANIMALS	MEAN, MINIMUM AND MAXIMUM VALUES			
	Total dose of dibenzanthracene given	Vitamin A per 100 gm. liver	Vitamin C per 100 gm. liver	Vitamin D per 100 gm. liver[2]
	mg.	*U.S.P. units*	*mg.*	*U.S.P. units*
Rats				
Normal (36)[1]	0	5884 (422–12,650)	26.8 (19.8–36.1)	37 (32–56)
Control (36)	0	6452 (516–13,574)	28.9 (20.0–33.8)	28 (21–45)
Treated (32)	30.1 (9–45)	28 (0–160)	28.3 (20.8–34.1)	32 (26–52)
Rabbits				
Normal (18)	0	2810 (914–4,265)	10.4 (7.4–17.1)	14 (11–18)
Control (18)	0	3188 (1096–5,274)	10.2 (7.0–16.4)	12 (8–20)
Treated (17)	72.2 (20–130)	36 (0–380)	11.0 (7.2–17.9)	10 (9–16)

[1] Numbers in parentheses indicate the number of animals observed.
[2] These vitamin D data pertain to groups of six animals.

ratio of mineral ash (A) to fat and organic material (R) ranged from 1.0 to 1.4 in the treated group as compared with an A/R ratio of 1.0 to 1.5 in the normal animals. In the treated rats the per cent of bone ash in the dry fat-free bones varied from 51.6 to 57.8 with an average of 55.3; in the normal animals, these values ranged from 52.6 to 56.8%, with an average of 54.8. The results of these experiments served to confirm those on vitamin D previously obtained, in which it was shown that dibenzanthracene had little effect on the storage of this compound in the liver.

The gelatin injections in the control group of animals did not affect the storage of any of the three vitamins, the rate of growth or the process of calcification.

SUMMARY

1. Intraperitoneal injections of 1,2,5,6-dibenzanthracene decreased the hepatic storage of vitamin A but not that of vitamins C or D. No vitamin A could be detected, however, in the urine excreted by the treated animals.

2. The inhibition of growth caused by dibenzanthracene in young rats was paralleled by the decrease in hepatic vitamin A.

3. No effect on the calcification of osseous tissues was observed in young rats treated with the hydrocarbon.

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THE INFLUENCE OF ARSENIC AND CERTAIN OTHER ELEMENTS ON THE TOXICITY OF SELENIFEROUS GRAINS [1, 2]

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THREE FIGURES

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Early investigations of the 'alkali disease' revealed that arsenic, vanadium, molybdenum, and chromium as well as selenium are present in some of the soils, feeds, and waters from the toxic areas (Beath et al., '35; Byers, '34; Miller and Byers, '35). Later studies on the comparative toxicities of selenium, tellurium, arsenic, molybdenum, tungsten, and vanadium (Franke and Moxon, '37) revealed that selenium was the only one of these elements to cause a severe pathological condition of the liver.

Since some of these elements occur with selenium in nature, it was of interest to determine what effect they might have on the toxicity of seleniferous grains. As a result of this study a method of counteracting selenium toxicity in laboratory animals has been developed.

EXPERIMENTAL

Sixty-five rats were divided into thirteen groups of five rats each. The groups were balanced as nearly as possible

[1] Published with the permission of the director of the Agricultural Experiment Station of South Dakota State College, Brookings, South Dakota.

[2] A preliminary report on part of this work was presented at the 96th Meeting of the American Chemical Society (Milwaukee, September, 1938) and also in Science, vol. 88, p. 81, 1938.

as regards weight and sex. The first twelve groups were fed a diet composed of:

	%
Seleniferous wheat (14 p.p.m. selenium)	82
Casein	10
Lard	3
Dehydrated yeast (Northwestern)	2
Cod liver oil	2
McCollum's salt mixture	1
	100

The diet contained 11 p.p.m selenium by analysis.

Each of eleven of the first twelve groups was given 5.0 parts per million (p.p.m.) of the following elements in their drinking water: tungsten, fluorine, molybdenum, arsenic, chromium, vanadium, cadmium, zinc, cobalt, uranium and nickel. An amount of a water-soluble salt (table 1) equivalent to 5.0 p.p.m. of each element was given in distilled water. Group no. 12 received distilled water without an added element. The thirteenth group, which served as a positive control, received distilled water and a diet similar to the one above except that control or selenium-free wheat was used in place of the seleniferous wheat.

Differences in weight and food consumption among the various groups were noted soon after the beginning of the experiment. Since the results were very significant at the end of 60 days a summary of the condition of the rats in the various groups at this time is included in table 1. At this stage of the experiment it appeared that fluorine, molybdenum, chromium, vanadium, cadmium, zinc, cobalt, and uranium caused an increase in the mortality rate. Since there were no controls which received these elements in the absence of selenium we cannot state whether the increased mortality rate was due to the element in the water or to the combined effect of selenium and the element. Very significant, however, was the fact that all rats in the tungsten and arsenic groups were living and normal in appearance at the end of 60 days.

At the end of 130 days the survivors (table 1) were killed and examined for liver damage. Those rats fed selenium

TABLE 1

GROUP	ELEMENT	AMOUNT	SALT USED	AVERAGE WEIGHT OF SURVIVORS AT 60 DAYS	SUMMARY AT 60 DAYS	AVERAGE WEIGHT OF SURVIVORS AT 130 DAYS	CONDITION OF SURVIVORS AT 130 DAYS
		p.p.m.		*gm.*			
I	W	5	$Na_2WO_4 \cdot 2H_2O$	70	All rats were living.	90	1 living (normal)
II	F	5	NaF	74	Three rats were dead at the end of 35 days. All showed necrosis of the livers, severe atrophy of central liver lobes, adhesions of liver to diaphragm and peritoneum, and internal hemorrhages.		All dead
III	Mo	5	$(NH_4)_2MoO_4 \cdot 2HOH$	107	Four rats dead at end of 40 days. All showed hemorrhages, atrophy and necrosis of livers.		All dead
IV	As	5	Na_2HAsO_3	115	Normal appearance. All living.	195	4 living; appear normal
V	Cr	5	$(NH_4)_2Cr_2O_7$	75	Four rats dead at end of 45 days. All had atrophy of central liver lobes and some showed necrosis of livers.		All dead
VI	V	5	$NaVO_3$	68	Four rats dead at end of 45 days. All had atrophy of central liver lobes, and necrotic and hemorrhagic livers.	132	1 living; rough coat
VII	Cd	5	$CdCl_2 \cdot 2\ 1/2\ H_2O$	106	Four rats were dead at end of 35 days. They all had necrosis and atrophy of the central liver lobes.		All dead
VIII	Zn	5	$ZnCl_2$		All five rats were dead at the end of 50 days. They had atrophy of the central liver lobes, severe necrosis, and hemachromatosis of the livers.		
IX	Co	5	$CoCl_2$		All five rats dead by the end of 35 days. They had atrophy of the central liver lobes and some necrosis of the livers.		
X	U	5	$UO_2(C_2H_3O_n)_2 \cdot 2H_2O$	103	Four rats were dead at end of 40 days. They had atrophy of central liver lobes and necrosis of the livers.		All dead
XI	Ni	5	$NiCl_2$	112	Two were dead at the end of 55 days. Both had necrosis and hemachromatosis of the livers, and atrophy of central liver lobes.	169	2 living; inactive; rough coat
XII	Se diet and distilled water			120	One rat died at the end of 45 days. It had internal hemorrhages, atrophy of central liver lobe, and hemachromatosis of the liver.	141	3 living; loss of hair; inactive
XIII	Control feed and distilled water			132	All rats normal.	201	All living and normal

alone, selenium with nickel, and the one survivor receiving selenium with vanadium, all had atrophied and necrotic livers, some of which exhibited hemachromatosis. The livers of the one survivor in the tungsten group, those fed arsenic with selenium, and the controls were normal in appearance. The livers were dried and analyzed for selenium. The results of the analyses together with the average total food intake, average total selenium intake and gain in weight of the survivors in each group are presented in table 2.

TABLE 2

GROUP	ELEMENT IN WATER	NUMBER SURVIVING AT 130 DAYS	AVERAGE GAIN PER RAT FOR 130-DAY PERIOD (survivors)	AVERAGE TOTAL FOOD INTAKE OF SURVIVORS	AVERAGE TOTAL SE INTAKE OF SURVIVORS	AVERAGE SE CONTENT OF LIVER p.p.m. dry weight	CONDITION OF LIVER
			gm.	*gm.*	*mg.*		
I	W	1	57	666	7.2	66	Normal appearance
IV	As	4	163.5	1277	14.2	26.8	Normal appearance
VI	V	1	99	1313	14.4	30	Atrophied Necrotic
XI	Ni	2	135	1207	13.2	52	Atrophied
XII	None	3	109	1032	11.3	45.5	Atrophied Necrotic Hemochromatosis
XIII	None	5	179	1389	None	0.0	Normal

Although the selenium-arsenic group consumed more of the diet and consequently had a greater intake of selenium, the concentration of this element in their livers was much lower than in the other groups receiving the seleniferous diet, with one exception. The one surviving rat in the group receiving vanadium had the highest selenium intake but had a relatively low concentration of selenium in the liver. However, all of the rats in the selenium-vanadium group showed typical symptoms of selenium poisoning.

The four rats in the tungsten group which did not survive the 130-day period died very near the end of the trial and at

autopsy none showed any appreciable liver damage. Also the one remaining rat of the group showed no gross liver damage although the liver did contain a high concentration of selenium. Group IV, those receiving arsenic in their water, grew normally, had good appetites and showed no liver damage when examined at the end of the experiment.

Since it appeared that tungsten and arsenic, especially the latter, reduced selenium toxicity in some manner, a second series was set up to determine whether or not lower concentrations of these elements would prevent selenium toxicity and also what effect these elements would have when given at these lower levels in the absence of selenium. Fifty-five rats were divided in groups as shown in table 3.

TABLE 3

GROUP	NUMBER OF RATS	DIET	DRINKING WATER
I	5	Control	Distilled water
II	10	Control	2.5 p.p.m. W in distilled water
III	10	Control	2.5 p.p.m. As in distilled water
IV	10	Seleniferous (11 p.p.m. Se)	Distilled water
V	10	Seleniferous (11 p.p.m. Se)	2.5 p.p.m. W in distilled water
VI	10	Seleniferous (11 p.p.m. Se)	2.5 p.p.m. As in distilled water

The experiment was continued for 75 days. The survivors were then autopsied and their organs examined.

The most important result of this trial was that 2.5 p.p.m. of arsenic in the drinking water would partially, but not completely, prevent liver damage in rats fed a diet containing 11 p.p.m. of selenium in the form of seleniferous wheat. Those rats given arsenic and a seleniferous diet consumed more food than those receiving the seleniferous diet and no arsenic. They also grew more rapidly and were much more active.

The same level of tungsten had no effect in preventing the characteristic symptoms of selenium poisoning. However, all ten rats lived the full length of the experiment while only seven of the ten fed the seleniferous diet and distilled water survived the trial. This reduction of mortality was the only

observed effect of tungsten toward decreasing selenium toxicity.

Neither arsenic nor tungsten at the level of 2.5 p.p.m. in the drinking water of rats receiving a control diet produced any change in food intake, growth, or general appearance from those receiving a similar diet and distilled water.

Water consumption was recorded throughout the trials in order to measure the amount of the added elements which the animals consumed, and also to note the effect of adding these various elements on the volume of water consumed during the entire experimental period. Table 4 shows the average water and element consumption for each group.

As is shown in table 4 there is no significant difference in the water consumption for any of the groups except the group receiving a seleniferous diet with no added element. The water consumption for this group was considerably lower, as is characteristic of the water consumption for selenized animals.

In the other trials in which 5 p.p.m. of arsenic were administered, there was no noticeable difference in the volume of water consumed, as compared with the series which received 2.5 p.p.m. arsenic. However, since the arsenic concentration was twice as great, the total amount of arsenic ingested was also twice as great, and, as has been explained, that amount afforded complete protection against the pathological condition caused by 11 p.p.m. of selenium.

To substantiate the results of the first trial, that 5.0 p.p.m. of arsenic as $NaHAsO_3$ would prevent the toxicity of a diet containing 11.0 p.p.m. of selenium as seleniferous wheat, a third trial was begun. Three groups of two rats each (one male and one female) were used. The first group was fed the control diet and received distilled water. Group 2 received distilled water and the seleniferous diet used in the previous trials. The third group was given the seleniferous diet also but received 5.0 p.p.pm. arsenic as sodium arsenite in its water.

TABLE 4

GROUP	ELEMENT ADDED TO WATER	AVERAGE DAILY WATER INTAKE IN ML PER RAT PER DAY	AVERAGE DAILY ELEMENT INTAKE IN MG.	AVERAGE TOTAL WATER INTAKE IN LITERS (75 DAYS)	TOTAL ELEMENT INTAKE IN MG. (75 DAYS)
Control diet + distilled water	None	Males—20.6 Females—15.65	None	Males—1.545 Females—1.173	None
Control diet + 2.5 p.p.m. W in water	W	Males—18.7 Females—16.7	Males—0.046 Females—0.041	Males—1.402 Females—1.252	Males—3.50 Females—3.13
Control diet + 2.5 p.p.m. As in water	As	Males—18.3 Females—16.2	Males—0.045 Females—0.0405	Males—1.372 Females—1.215	Males—3.43 Females—3.03
Seleniferous diet + distilled water	None	Males—14.8 Females—12.8	None	Males—1.110 Females—0.960	None
Seleniferous diet + 2.5 p.p.m. W in water	W	Males—12.9 Females—13.8	Males—0.032 Females—0.034	Males—0.967 Females—1.035	Males—2.41 Females—2.58
Seleniferous diet + 2.5 p.p.m. As in water	As	Males—19.0 Females—18.9	Males—0.047 Females—0.047	Males—1.425 Females—1.417	Males—3.562 Females—3.542

The external condition of the rats after being on the experiment for 60 days is shown in figure 1. Those fed the toxic diet and distilled water gained weight very slowly, were inactive, showed loss of hair and reduced food consumption. In contrast, those of the third group which received arsenic in their drinking water and the seleniferous diet showed none

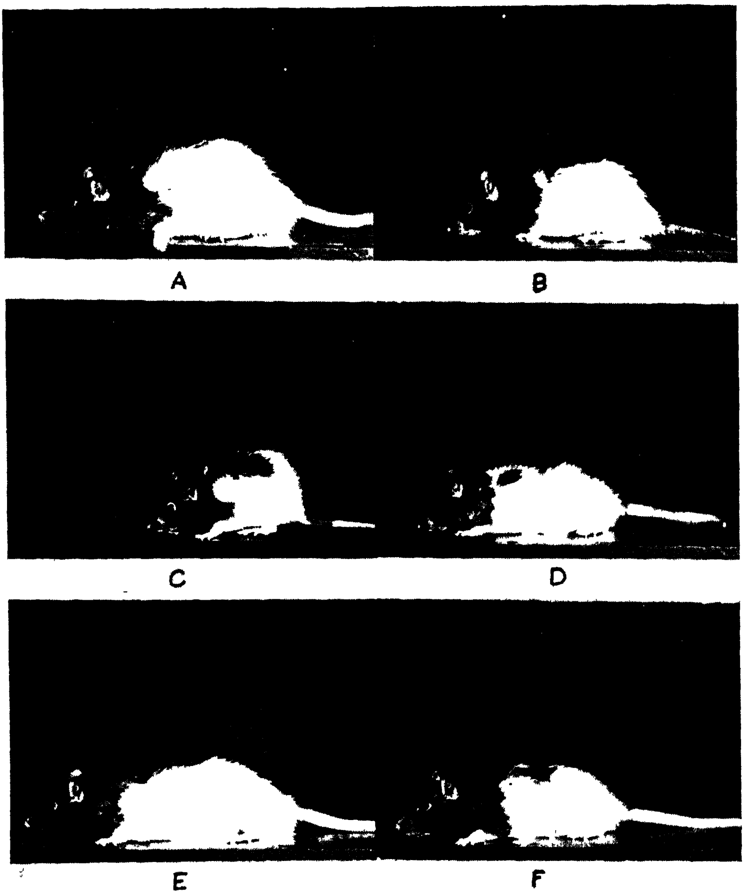

Fig. 1 A, C and E, males; B, D and F, females. A and B, control diet + distilled water; C and D, seleniferous diet (11.0 p.p.m. Se) + distilled water; E and F, seleniferous diet (11.0 p.p.m, Se) + distilled water containing 5 p.p.m. of arsenic as Na_2HAsO_3.

of these symptoms. Their food consumption was normal and their growth paralleled that of the control animals as is shown in figure 2.

Since both of the rats on the seleniferous diet were dead by the sixty-fifth day of the trial the survivors were killed on the seventieth day of the experiment. The condition of their livers at the time of death is shown in figure 3. While

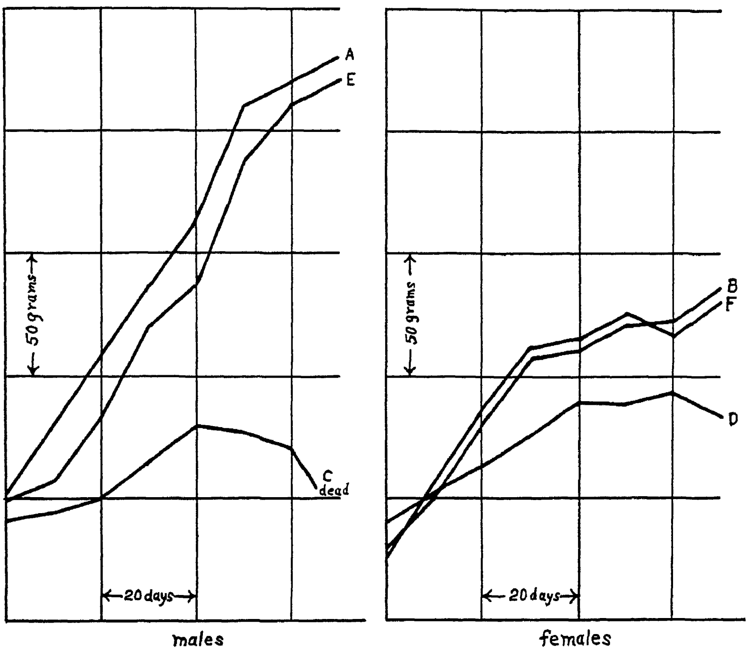

Fig. 2 Growth curves of animals shown in figure 1.

the livers of both rats on the seleniferous diet (C and D) were severely damaged, the livers from the rats getting arsenic and a seleniferous diet (E and F) showed no liver damage at all. This again indicated that arsenic, in some manner, reduced the toxicity of seleniferous wheat when fed to rats. A report of further work on the counteracting effect of arsenic on the toxicity of various forms of selenium is in preparation.

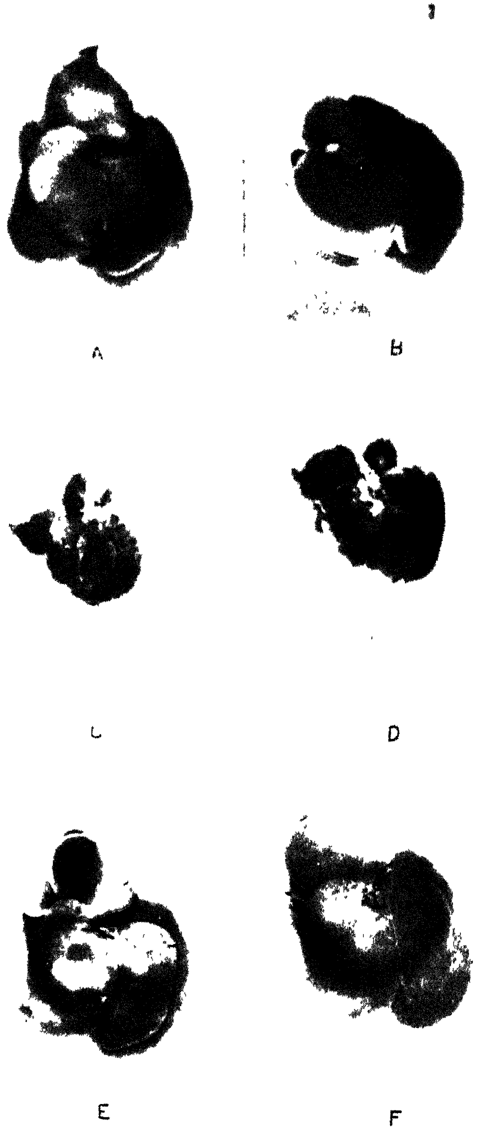

Fig. 3 Livers from corresponding animals in figure 1.

SUMMARY AND CONCLUSIONS

Five p.p.m. of fluorine, molybdenum, chromium, vanadium, cadmium, zinc, cobalt, nickel, and uranium given as water-soluble salts in the drinking water of rats fed a diet containing 11 p.p.m. of selenium from seleniferous wheat caused an increase in mortality.

The same level of tungsten in the form of $Na_2WO_42H_2O$ in the water prevented, to some extent, the typical liver damage caused by the seleniferous diet, and in addition seemed to decrease mortality of rats on such a diet.

Two and one-half p.p.m. of tungsten in the drinking water seemed to reduce the mortality rate of rats fed the seleniferous diet but did not prevent liver damage.

Complete prevention of the symptoms of selenium poisoning was accomplished by giving 5 p.p.m. of arsenic as sodium arsenite in the drinking water.

Two and one-half p.p.m. of arsenic prevented, to some extent, the selenium poisoning symptoms ordinarily caused by a diet containing 11 p.p.m. of selenium from seleniferous wheat. This level of arsenic did not prevent liver damage appreciably, but did bring about a greater food intake and more nearly normal growth.

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THE MINIMUM VITAMIN-A REQUIREMENTS OF NORMAL ADULTS

II. THE UTILIZATION OF CAROTENE AS AFFECTED BY CERTAIN DIETARY FACTORS AND VARIATIONS IN LIGHT EXPOSURE [1]

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ONE FIGURE

(Received for publication July 21, 1939)

Results reported in the first paper of this series (Booher, Callison, and Hewston, '39) had shown that unit for unit commercial carotene dissolved in cottonseed oil was only about 50 to 60% as efficient in supporting normal dark adaptation in adults as vitamin A in the form of cod liver oil. The ratio of the carotene requirement to the vitamin-A requirement, as determined for five subjects, varied within the rather narrow limits of 1.84 and 1.61.

Wilson, Das Gupta and Ahmad ('37) found that the carotene in raw carrots and in cooked spinach (sak) was about 80 to 90% absorbed by a normal adult when the diet contained an ordinary quantity of fat; when fat was excluded from the diet, however, the absorption of carotene from cooked spinach was reduced to about 50%.

Van Eekelen and Pannevis ('38) reported the carotene in cooked spinach and in cooked carrots to be absorbed only to the extent of 6 and 1%, respectively, when fed to adults in conjunction with a diet of bread, rice, lard, meat, marmite, and 50 mg. of vitamin C. Under similar conditions these workers found that beta carotene dissolved in oil was absorbed to the extent of about 59%.

[1] This project was supported by an appropriation from Bankhead-Jones Funds (Bankhead-Jones Act of June 29, 1935).

Carotene and vitamin-A balance studies are complicated by difficulties in interpretation and by lack of satisfactory methods for recovering these substances from excreta. In order to obtain practical information on the utilization of the carotene in vegetables by human subjects, determinations were made of the physiologic minimum vitamin-A requirements of adults in terms of commercial crystalline carotene and in terms of the carotene as it occurs in peas and spinach. Coincident with these measurements investigations were made on the influence of controlled light exposure and of increased dietary fat, vitamin B_1 and riboflavin in modifying the carotene requirement or utilization of the carotene in vegetables.

EXPERIMENTAL

The details of our method for determining the physiologic minimum vitamin-A requirements of adults and for managing the dietary regimen of the experimental subjects have already been described (Booher, Callison, and Hewston, '39). As in the case of our previous studies the dark adaptation measurements were made with the visual adaptometer described by Hecht and Shlaer ('38).

Chromatographic analyses [2] of the crystalline commercial carotene used in the experiments herein described indicated that it contained 14.0×10^3 mg. and 79.4×10^3 mg. of alpha and beta carotene respectively per 100 gm. Such composition would, presumably, correspond to 1.44×10^8 international units of vitamin A in 100 gm. of the carotene. According to biological assays,[3] using U.S.P. reference cod liver oil as a standard, the carotene contained 1.67×10^8 international units per 100 gm.

Both the peas and the spinach were frozen products purchased at one and the same time in sufficient numbers of 5 pound lots to last throughout the entire investigation. The

[2] We are indebted to L. A. Shinn, Bureau of Dairy Industry, U. S. Department of Agriculture for the chromatographic analyses.

[3] We are indebted to Rosemary Loughlin, for valuable assistance in making the biological assays.

average percentages of dry matter in five samples of the frozen peas and spinach taken from five different lots of each vegetable were 23.5 ± 1.6 and $8.5 \pm 0.9\%$, respectively.

According to chromatographic analyses [4] the peas carried 0.37 mg. and the spinach 5.1 mg. of beta carotene per 100 gm. (raw, fresh weight basis) thus corresponding to 620 and 8500 international units, respectively, of vitamin A per 100 gm. The chromatographic analysis of the carotene in each vegetable was made on a composite sample made up of equal weights of the 5 pound lots of peas and spinach, respectively.

The biological assays [5] for vitamin A-values of the peas and spinach after cooking, using U.S.P. reference cod liver oil as the standard, were 700 and 9500 to 10,000 units, respectively, per 100 gm. (raw basis) of the vegetables. The vitamin-A assays of the vegetables were conducted in parallel with the determinations of the minimum vitamin-A requirements of adults in terms of these same vegetables. The thrice weekly supplements of the green peas and of the spinach fed to the vitamin-A assay rats were taken in successive order from the 5 pound lots of each vegetable.

The vitamin-A values of the cooked peas and cooked spinach are, within the limits of experimental error, in excellent agreement with the vitamin-A values determined by chromatographic analyses.

HUMAN UTILIZATION OF THE CAROTENE IN COOKED PEAS AND SPINACH

The vitamin-A values of the minimum daily intakes of carotene dissolved in cottonseed oil, of cooked peas, and of cooked spinach, which will just support normal dark adaptation in adults, are shown in table 1. In order that comparison of these requirements with those similarly determined for vitamin A in the form of cod liver oil may be facilitated, the results on our five subjects reported earlier are included in this same table. In every case the utilization of the vitamin-A value of the cooked vegetables was superior to that of the

[4] See footnote 2, page 460.

[5] See footnote 3, page 460.

TABLE 1

Summary of minimum vitamin-A requirements of adults in terms of cod liver oil, crystalline carotene, cooked peas, and cooked spinach

SUBJECT	VITAMIN A IN COD LIVER OIL		CAROTENE IN COTTONSEED OIL		RATIO: CAROTENE REQUIREMENT TO VITAMIN-A REQUIREMENT	CAROTENE IN COOKED PEAS		RATIO: CAROTENE REQUIREMENT TO COOKED PEA REQUIREMENT	CAROTENE IN COOKED SPINACH		RATIO: CAROTENE REQUIREMENT TO COOKED SPINACH REQUIREMENT
	Units/kg.	Total units [1]	Units/kg.	Total units [1]		Units/kg.	Total units [1]		Units/kg.	Total units [1]	
H. W.	35.7	1890	57.4	3040	1.61	....	....	....	....	....	....
M. P.	55.8	3900	102.6	7180	1.84	....	....	....	....	....	....
D. E.	52.4	3140	90.0	5400	1.72	....	....	....	....	....	....
H. E.	24.8	1290	42.5	2210	1.71	....	....	....	....	....	....
C. E.	34.3	2060	60.5	3630	1.76	....	....	....	....	....	....
R. E.	....	....	76.3	5180	....	47.0	3200	1.62	....	....	....
B. F.	....	....	96.3	5680	....	57.3	3380	1.68	77.4	4570	1.24
C. C.	....	....	106.2	6795	....	....	....	....	87.0	5570	1.22
C. L.	....	....	....	....	....	....	....	....	101.0	4350	....

[1] The figures in this column include the vitamin-A content of the basal diet plus the vitamin-A value of the supplements.

utilization of the crystalline carotene in cottonseed oil. The ratios of the vitamin-A requirements when fed in the form of commercial crystalline carotene dissolved in cottonseed oil to the requirements supplied in the form of carotene in cooked peas were 1.66 and 1.62 for the two subjects, respectively. When cooked spinach was used in place of cooked peas, these ratios for two subjects were 1.24 and 1.22 respectively. It is apparent, therefore, that the carotene in cooked peas is better utilized than the carotene in cooked spinach. According to the data shown in table 1, it may be concluded that the utilization of the vitamin-A values of cooked peas and cooked spinach is intermediate between the utilization of the vitamin-A value of cod liver oil and that of crystalline carotene in cottonseed oil. Actually the utilization of the vitamin-A value of cooked peas approaches that of cod liver oil while the utilization of the vitamin-A value of cooked spinach is more nearly like that of crystalline carotene dissolved in cottonseed oil.

The average composition of the experimental diets for three of the subjects, for whom the minimum physiologic requirements for vitamin A in terms of cooked peas and of cooked spinach were determined, is summarized in table 2. The data shown graphically in figure 1 represent the general type of dark adaptation response encountered in measurements of the minimum physiologic requirements for vitamin A in terms of a specific food item, in this case cooked green peas.

UTILIZATION OF THE VITAMIN-A VALUE OF CAROTENE AS INFLUENCED BY CERTAIN DIETARY FACTORS AND BY VARIATIONS IN LIGHT EXPOSURE

From time to time various dietary factors have been mentioned as potential promoters of carotene or vitamin-A utilization. Increased intake of fat or of thiamin are the most frequently mentioned of these dietary factors suggested as promoting the utilization of carotene.

The observation of Aykroyd ('30) to the effect that fishermen in Labrador conserve their ability to see in dim light by keeping one eye shielded from light during the day suggests

TABLE 2

Average composition of experimental diets of three subjects for whom the minimum physiological requirements for vitamin A in terms of cooked peas and of cooked spinach were determined. Quantities refer to daily intakes

DESCRIPTION OF EXPERIMENTAL PERIODS	TOTAL CALORIES	PROTEIN	FAT	MINERALS			VITAMINS			
				Ca	P	Fe	A	Thiamin	Ascorbic acid	Riboflavin
Subject B. F.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*I.U.*[1]	*I.U.*	*I.U*	*S.U.*[2]
1. Depletion period	1840	77	58	1.0	1.3	0.026	80	480	1830	610
2. Vitamin-A deficient diet + carotene in oil	1970	71	64	1.2	1.4	0.024	5680	450	1730	590
3. Depletion period	1760	74	57	1.1	1.4	0.023	70	450	2140	600
4. Vitamin-A deficient diet + cooked peas[3]	1910	87	48	1.2	1.7	0.027	3380	800	2930	710
5. Vitamin-A deficient diet + cooked spinach	1700	77	49	1.1	1.4	0.024	4570	400	2340	580
Subject R. E.										
1. Depletion period	2380	90	83	1.2	1.5	0.026	80	550	2020	650
2. Vitamin-A deficient diet + carotene in oil	2400	115	88	1.2	1.8	0.030	5180	600	2000	670
3. Depletion period	2390	96	71	1.0	1.4	0.028	70	630	2000	600
4. Vitamin-A deficient diet + cooked peas	2530	108	81	1.0	1.9	0.031	3200	1000	2530	710
Subject C. C.										
1. Depletion period	1780	71	52	1.1	1.3	0.024	80	440	2210	590
2. Vitamin-A deficient diet + carotene in oil	1760	73	46	1.1	1.4	0.023	6795	480	2240	600
3. Depletion period	1760	76	30	1.2	1.4	0.024	75	500	2420	580
4. Vitamin-A deficient diet + cooked spinach	1650	72	44	1.1	1.3	0.025	5570	460	3580	610

[1] International units.

[2] Sherman-Bourquin units.

[3] A short period on the vitamin-A deficient diet alone was interposed between periods 4 and 5 during which the composition of the diet was very similar to those given for periods 1 and 3.

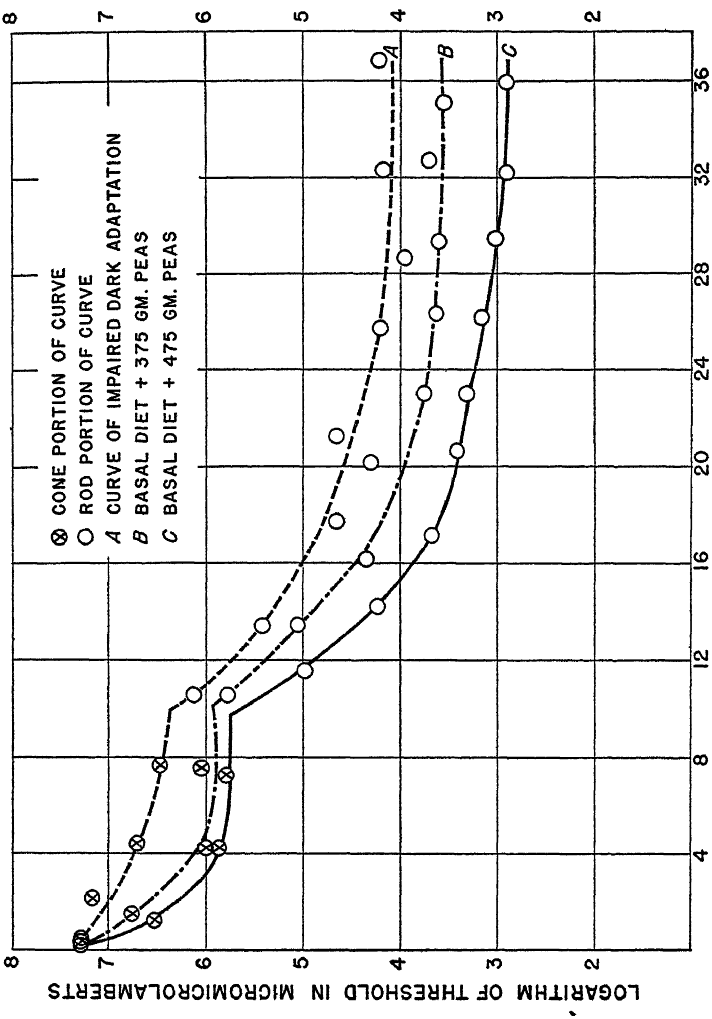

Fig. 1 Dark adaptation curves for subject B. F., showing recovery of normal dark adaptation with addition of cooked peas to the diet.

the possibility of light exposure having an appreciable influence on vitamin-A requirements.

The presence of riboflavin in the retinas of cattle, sheep, dogs, chickens and fish was reported by Euler and Adler ('34). Adler and Euler ('38) have suggested that the free riboflavin present in high concentration in the fish retina may take part in the visual processes by virtue of its sensitivity to light. Chase ('37) has lent support to this theory by his discovery that solutions of frog's visual purple bleached by blue light regenerates more quickly than when bleached by yellow light, indicating the presence of a blue-sensitive substance which influences this regeneration. However, direct evidence in support of riboflavin being concerned along with vitamin A in the processes of dim vision has never been advanced.

In view of the possibility that one or more of these factors might alter the interpretation of the results on which our estimates of the minimum physiologic requirements for vitamin A have been based, the influence of each of the above-mentioned factors was investigated in turn. The summary of the experimental plan followed in this investigation and the general type of results obtained are shown in table 3. On account of the limitations of space, it was necessary to limit presentation of actual data in table 3 to the minimum that would show the method of procedure with an example of the results obtained with each factor investigated.

In order to make any effects of these variants stand out as sharply as possible, they were, in each case, superimposed upon a given daily intake of carotene that was less than was required for complete restoration of normal dark adaptation. When the partial recovery from hemeralopia became established and steady over a period of several days the experimental variants under investigation were superimposed and the effects on the dark adaptation curve of the subject were determined over another period of several days. Under these conditions any improvement in the dark adaptation response would indicate better utilization of the carotene as a result of the superimposed experimental variant.

TABLE 3

Data showing the influence of variations in intake of thiamin, riboflavin and dietary fat and of light exposure on vitamin A (supplied as carotene) requirements of adults

SUBJECT AND EXPERIMENTAL CONDITIONS [1]	DURATION OF EXPERIMENTAL PERIOD	AVERAGE LIGHT THRESHOLDS [2]	
		After 6 minutes dark adaptation	After 30 minutes dark adaptation
B. F. (female) age 32; height 164 cm.; weight 59 kg.:	*days*	*log $\mu\mu$ lamberts*	*log $\mu\mu$ lamberts*
Before depletion	....	5.67 ± 0.21	2.96 ± 0.08
Vitamin-A deficient diet	48	6.11	4.00
Vitamin-A deficient diet + carotene (3300 units vitamin A)	4	5.77 ± 0.06	3.33 ± 0.02
Vitamin-A deficient diet + carotene (3300 units vitamin A) + 170 μ grams thiamin	14	6.02 ± 0.06	3.51 ± 0.10
Vitamin-A deficient diet + carotene (5605 units vitamin A)	4	5.70 ± 0.05	3.03 ± 0.09
C. L. (female) age 19; height 155 cm.; weight 43 kg.:			
Before depletion	....	5.72 ± 0.15	3.17 ± 0.12
Vitamin-A deficient diet	22	6.20	4.20
Vitamin-A deficient diet + cooked spinach (1900 units vitamin A)	14	5.95 ± 0.19	3.69 ± 0.66
Vitamin-A deficient diet + cooked spinach (1900 units vitamin A) and wearing goggles in daytime	3	5.95 ± 0.06	3.48 ± 0.07
Vitamin-A deficient diet + cooked spinach (1900 units vitamin A) + 200 μ grams thiamin + 300 μ grams riboflavin	4	6.00 ± 0.00	3.46 ± 0.08
Vitamin-A deficient diet + cooked spinach (3325 units vitamin A)	7	5.94 ± 0.07	3.33 ± 0.05
Vitamin-A deficient diet + cooked spinach (3325 units vitamin A) and with 6 hours daily exposure to bright sunshine	5	5.92 ± 0.08	3.29 ± 0.10
Vitamin-A deficient diet + cooked spinach (4275 units vitamin A)	8	5.76 ± 0.04	3.02 ± 0.02
Y. E. (male) age 19; height 178 cm.; weight 67 kg.:			
Before depletion	....	5.86 ± 0.18	3.25 ± 0.18
Vitamin-A deficient diet (35% of total day's calories from fat)	40	6.15	3.70
Vitamin-A deficient diet + carotene (4355 units vitamin A)	16	5.83 ± 0.10	3.31 ± 0.13
Vitavin-A deficient diet + carotene (4355 units vitamin A) with proportion of calories from fat increased to 40%	15	6.18 ± 0.17	3.50 ± 0.00
Vitamin-A deficient diet + carotene (5025 units vitamin A)	21	5.90 ± 0.08	3.16 ± 0.18

[1] All dietary supplements mentioned are per diem.

[2] Thresholds were measured at 7° on nasal side of fovea, image a 3° test spot. The values for subjects before depletion are averages of 9 to 21 measurements taken on different days. The second set of threshold values for each subject is for the last day of the depletion period. The remaining thresholds are representative averages of determinations made toward the latter part of the periods described; the measurements, in each case, were made 18 to 22 hours subsequent to the administration of the previous day's vitamin supplements.

According to the results shown in table 3 it would appear that a daily intake of thiamin in excess of 400 to 600 international units (see table 2 for thiamin values of the basal vitamin A-deficient diets) did not improve the utilization of carotene. In addition to the results shown for subject B. F., the findings were confirmed by results obtained with a male subject with whom the same experimental plan was carried out. The dark adaptation response of these two adults already receiving daily around 60% of the physiologic minimum requirement of carotene necessary for the support of normal dark adaptation was not improved as a result of increased intakes of thiamin. Neither did enrichment of the diet in thiamin and riboflavin together show any improvement in the average light threshold values (see data on subject C. L., table 3) when superimposed upon a supplement of cooked spinach that was inadequate to support fully normal dark adaptation. The vitamin A-deficient basal diet of this subject previous to enrichment with the 300 micrograms of pure riboflavin carried about 600 Sherman-Bourquin units of vitamin G (probably equivalent to around 1800 to 2400 micrograms of riboflavin) per day. The cooked spinach supplements used as a source of vitamin A were consumed by the subject in equal portions at the noon and evening meals.

The average fat content of the vitamin A-deficient diets of the experimental human subjects was such that approximately 30 to 35% of the total calories were provided in the form of fat. This level of dietary fat corresponds to that ordinarily found in average American dietaries. As shown in table 3 (see data on subject Y. E. as the example) increase in fat intake beyond this level had no appreciable effect upon the utilization of carotene. This observation was confirmed by results similarly obtained for a young adult female subject.

One subject (see data for subject C. L., in table 3) submitted for a 3-day period to wearing closely fitted dark goggles of the type frequently used to protect the eyes from the intense light of high voltage welding-arcs. The transmission of the darkened glass was approximately 2%. The goggles

were worn steadily with the exception of about 1 hour a day during the entire waking period of the 3-day interval. Preliminary to each measurement of dark adaptation during the 3-day period this subject was adjusted gradually to ordinary indoor daylight before proceeding with the visual measurements. It was not feasible for us to continue this variation in experimental conditions beyond this one 3-day interval and we have not, to date, had the opportunity to repeat the experiment with other subjects. However, from the observation of this one case it would appear that if any lowering of the vitamin A requirement attended the diminution of exposure of the eyes to ordinary daylight, its magnitude is so small as to be of no practical significance in defining minimum vitamin A requirements.

After a 7-day period of increased but inadequate intake of carotene in the form of spinach, this same subject was exposed to bright outdoor sunshine for 6 hours daily over a period of 4 days. This exposure to light was without appreciable effect on the threshold values measured during the course of dark adaptation. These results are in accord with the fact that maintenance of a normal visual purple mechanism represents only a very small part of the physiologic need of the body for vitamin A.

SUMMARY

The vitamin A assay values of cooked peas and cooked spinach, as determined by the rat-growth method using U.S.P. reference cod liver oil as the vitamin A standard, were found to be in excellent agreement with the carotene analysis of spinach as determined by chromatographic analysis.

A daily intake of approximately 47 and 57 units of vitamin A, respectively, per kilogram of body weight was necessary for the maintenance of normal dark adaptation in two normal adults when the vitamin A value of the diet was derived almost entirely from the carotene in cooked green peas.

A daily intake of approximately 77, 87 and 101 units of vitamin A, respectively, per kilogram of body weight was

required for the same response in three adults when the vitamin A value of the diet was derived almost entirely from the carotene in cooked spinach.

For maintenance of normal dark adaptation in adults the utilization of the vitamin A values in cooked peas and cooked spinach is intermediate between those for cod liver oil and for commercial crystalline carotene dissolved in cottonseed oil. The utilization of the vitamin A value of cooked peas was better than that of cooked spinach.

A daily intake of thiamin in excess of 400 to 600 international units does not appear to improve the utilization of carotene by adults nor to diminish their minimum physiologic requirement for vitamin A.

A daily intake of riboflavin in excess of about 1800 to 2400 micrograms was not accompanied by increased utilization of carotene nor by diminution of the minimum physiologic requirement for vitamin A in adults.

Dietary fat in an ordinary mixed diet in excess of that which provides about 30 to 35% of the total caloric intake showed no beneficial effects upon the utilization of carotene by normal adults.

A marked increase or a marked decrease in exposure of the eyes of normal adults to ordinary light sources is probably not accompanied by significantly altered requirements for vitamin A.

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We wish particularly to acknowledge our indebtedness to Edna Mohagan for her invaluable contribution in planning and preparing the meals for the experimental subjects and in making the daily calculations of food intakes; to Pearl Anderson and Virginia Knowles who assisted with the dark adaptation tests; and to those of our associates in the bureau of home economics who served as subjects for these experiments.

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BASAL METABOLISM OF THE ADULT RABBIT AND PREREQUISITES FOR ITS MEASUREMENT

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ONE FIGURE

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The unusually large range in the basal heat production of the rabbit, as reported by previous investigators who give heat values differing by more than 100%, has challenged us to investigate this subject further. As already pointed out (Smuts, '35; Friedgood and Bevin, '39), the conditions prerequisite for basal metabolism measurements on the rabbit have not been well defined; the statements concerning these conditions are conflicting, and no comprehensive study of the basal metabolism of this animal has been made. Because adult rabbits have a sevenfold range in weight and yet are of comparable proportions, they offer ideal conditions to study the specific effect of size upon metabolism within the same species. Hence this investigation was undertaken with three objectives in view, 1) to determine the conditions necessary for basal metabolism measurements on the rabbit, 2) to establish this animal's basal metabolic level, and 3) to study the influence of size on heat production within the same species. The first two of these objectives will be discussed in this report. The third will be considered in a subsequent paper.

TECHNIC

The metabolism was measured with two forms of respiration apparatus, both on the open-circuit principle. For 3- to

7-kg. rabbits, the volume of ventilating air was measured by dry gas meters recording to 0.1 liter, and for 1- to 3-kg. rabbits, by a wet gas meter recording to 0.01 liter. Aliquot samples were collected in 1-liter spirometers for the small rabbits and in the Fox bag (Carpenter and Fox, '31; Benedict, '36) for the large rabbits. The samples of ventilating air were analyzed on the apparatus of Carpenter ('33). The size of chamber varied according to the size of the animal, its capacity ranging from 5.0 liters for a 1-kg. rabbit to 16 liters for a 7-kg. rabbit. Constancy (usually within 1%) in the ventilation rate from period to period was maintained by means of a Murrill (1898) regulator.

The apparatus were frequently tested for tightness. In alcohol control tests the respiratory quotients ranged from 0.644 to 0.669 in the individual periods of measurement. The average ratio in seven experiments with the larger apparatus was 0.660 and in four experiments with the smaller apparatus, 0.659. The gas-analysis apparatus was checked on nearly every experimental day by analyses of outdoor air. These analyses (119) showed a range in carbon dioxide of from 0.029% to 0.033% (average 0.030%), and in oxygen of from 20.934% to 20.948% (average 20.939%), with standard deviations of ± 0.001 and ± 0.003, respectively.

The chamber temperature (indicated by a mercurial thermometer, with its bulb in the outgoing air pipe at its exit from the chamber) was held at the desired level by placing the chamber in a larger, water-jacketed chamber, which provided several inches of air space between the water jacket and the metabolism chamber. Thermostatic control was frequently used to maintain the temperature of the animals' room within the desired limits, and the temperature was recorded by a calibrated thermograph. Body temperature was measured by thermocouples (Lee, '39).

In all experiments graphic records of activity were obtained by placing the chamber on a platform, suspended by a pneumograph.

PREREQUISITES FOR BASAL METABOLISM MEASUREMENTS

The conditions necessary for basal metabolism measurements on any animal species have been pointed out independently by Benedict ('38) and Giaja ('38). Both emphasize absence of muscular activity, post-absorptive condition, zone of thermic neutrality, adaptation to this zone, normal nutritive state, the animal's age (avoidance of the complicating factors of growth and senility), and time of day at which the measurement is made. Le Breton ('34 a) has partially set forth the conditions for determination of the rabbit's basal metabolism. The conflicting data and statements of several careful investigators, however, concerning the zone of thermic neutrality and the time when the post-absorptive condition is reached leave uncertainty as to these factors.

Animals used. Seventy-five rabbits were obtained from three sources. One group was obtained from local breeders. Two other groups were lent to us, one through the kindness of Prof. William E. Castle of the Bussey Institution of Harvard University, and the other through the kindness of Prof. Gregory Pincus of the Department of Physiology of Harvard University. Rabbits 10, 40, 47, 52, and 62 were 7 to 9 months old; rabbit 36 was 5 years old, and the others ranged in age from 10 months to 4 years at the time of their first metabolism measurements. The average age was 1⅔ years, and the maximum age in any experiment, 6 years. As the rabbit's life span may be at least 10 years (Tegge, '36), and as rabbits mature sexually at about 6 months (Fangauf and Immenkamp, '38), our rabbits were all adult but in no sense senile. They were also in good nutritive state, as indicated by their general appearance, by dissection of a number of animals, and by comparison of their body weights with standard weights for respective breeds. Their weights ranged from 1 to 7 kg. They were housed in individual cages and, unless fasting for experiments, were fed each morning Purina rabbit chow, supplemented frequently by carrots, carrot tops, and parsley.

Activity. As excessive activity can double the rabbit's metabolism, this factor has been eliminated in the following

discussions of results in two ways. Only measurements in periods when no appreciable activity occurred have been considered, and of these, only period values within 10% of the minimum value found in any experiment were included in the average for the experiment. Usually each average value represents from three to five periods of measurement, occasionally only two periods or all six periods.

Post-absorptive state. The two criteria for establishing absence of digestive activity are the respiratory quotient and the heat production. An analysis of the respiratory quotients (measured at approximately 28°C.) according to the length of time food had been withheld from the rabbits reveals that a 24-hour fast did not always result in quotients of fat. The 165 values at the twenty-fourth hour after food ranged from 0.67 to 0.86, but the greatest number were 0.74, which was the average value. At the forty-eighth hour the nineteen quotients ranged from 0.67 to 0.74 and averaged 0.71. The fifteen quotients at the seventy-second hour also averaged 0.71, and the range was from 0.68 to 0.73. In eleven experiments (also at about 28°C.), in which the exact time of the last food ingestion was known, the maximum quotients within 6 hours of this time varied from 0.80 to 0.96, over half of them being 0.90 or above. The ranges and averages at the twenty-fourth hour (eleven experiments), forty-eighth hour (nine experiments), and seventy-second hour (five experiments) of fasting were as follows:

Hours fasting	*Range*	*Average*
24	0.70 to 0.79	0.74
48	0.68 to 0.75	0.71
72	0.68 to 0.73	0.70

Comparison of the heat production and the simultaneously determined respiratory quotient at various stages of fasting is given in table 1 for sixteen experiments, most of them at about 28°C. The heat production had only a slight tendency to decrease as the fast progressed and, on the whole, was essentially the same whether the rabbit had been fasting 24, 48 or 72 hours. Exceptions are noted in experiments e, g, i and

m, in which the heat production decreased between the twenty-fourth and forty-eighth hours, although quotients at the fat level were found at the twenty-fourth hour. After 24 hours of fasting there was no consistent tendency for a decreasing respiratory quotient to be accompanied by a lowered heat production. In experiments a, c, f and k the respiratory quotient decreased as fasting continued from the twenty-

TABLE 1

Relation of heat production per square meter of body surface[1] per 24 hours and respiratory quotient to length of fast

EXPERIMENT[2]	RABBIT NO.	WEIGHT	HOURS FASTING							
			1 to 6 hours		24 hours		48 hours		72 hours[3]	
			R. Q.	Calories	R. Q.	Calories	R. Q.	Calories	R. Q.	Calories
		kg.								
a	9	1.39	0.80	807	0.76	602	0.72	724		
b	16	4.00	0.92	892	0.77	821	0.70	[4]	0.73	812
c	55	4.00	0.91	1026	0.77	753	0.72	748		
d	2	1.14			0.68	522	0.72	528	0.70	499
e	2	1.16	0.78	723	0.70	605	0.72	477	0.72	503
f	2	1.20			0.74	518	0.70	520	0.70	499
g	9	1.44	0.80	648	0.71	580	0.67	557	0.70	544
h	9	1.35			0.72	553	0.71	562	0.71	541
i	9	1.34			0.70	624	0.72	580	0.70	547
j	12	1.61			0.72	601	0.71	589	0.70	623
k	65	3.55			0.76	632	0.73	676	0.69	623
l	47	3.76			0.72	652	0.71	642	0.70	617
m	63	4.77	0.79	857	0.70	856	0.70	829	0.68	923
n	63	4.56			0.73	858	0.69	885	0.72	817
o	63	4.28			0.73	810	0.70	829	0.71	781
p	68	5.15			0.74	551	0.70	543	0.69	560
Average[5]					0.72	643	0.71	632	0.70	621

[1] Calculated from formula $S = 0.001w \left(\frac{K \times w^{2/3}}{10{,}000}\right)$ in which S represents square meters; w, weight in grams, and K = 10.

[2] The rabbits were kept at essentially the experimental temperature for 24 hours prior to the first measurements and also between measurements; all experiments were at 27° to 32°C. except experiments a, b, c and k which were at 24°C.

[3] Observations at the ninety-sixth hour gave the following results: experiment i, R. Q. = 0.69, 567 cals.; experiment j, R. Q. = 0.69, 604 cals.; experiment o, R. Q. = 0.70, 727 cals.

[4] Active.

[5] Not including experiments a, b and c.

fourth to the forty-eighth hour, but the level of the heat production was not lowered. There were also experiments (a and k) when the minimum or approximately the minimum heat production occurred with the highest respiratory quotient. Thus the minimum heat production is not dependent on or insured by a respiratory quotient of fat. In table 1 the average heat production at the seventy-second hour was only 3% below that at the twenty-fourth hour. At the ninety-sixth hour the heat production in two of three experiments was the same as at the seventy-second hour. Hence, between the twenty-fourth and the seventy-second hours of fasting, any effect of absorption of food on the metabolism can be considered negligible and the heat production can be considered to be at a level, even though the respiratory quotient may not be that of fat.

A large number of experiments revealed that the respiratory quotient does not change significantly in the course of 2 to 3 hours, when the rabbit has been fasting at 27° to 28°C. for 22 to 26 hours. In representative experiments, each of six 25- to 30-minute periods, the following quotients were noted:

Rabbit no.	*Period no.*					
	1	2	3	4	5	6
3	0.73	0.72	0.72	0.73	0.73	0.72
23	0.71	0.70	0.72	0.70	0.72	0.71
24	0.71	0.71	0.72	0.72	0.73	0.73
42	0.73	0.75	0.74	0.74	0.76	0.73

As the quotients did not vary significantly in any one experiment, it is concluded that the average of any two individual quotients in a given experiment will be representative of all the quotients. Therefore the respiratory quotient was determined usually in only two (non-consecutive) periods of the six periods of each basal experiment, and the two quotients were averaged. In the other periods only the carbon-dioxide production was measured. The heat production in the different periods was then calculated by applying the caloric value of carbon dioxide for this average respiratory quotient

to the measured carbon-dioxide production, reduced to standard conditions of temperature and pressure.

Zone of thermic neutrality and period of habituation to temperature. In figure 1, curves are given showing the course of the heat production per square meter of surface area of rabbit 12 as the environmental temperature was increased. The animal had been fasting 24 hours at the start of each experiment and remained at each temperature level for about

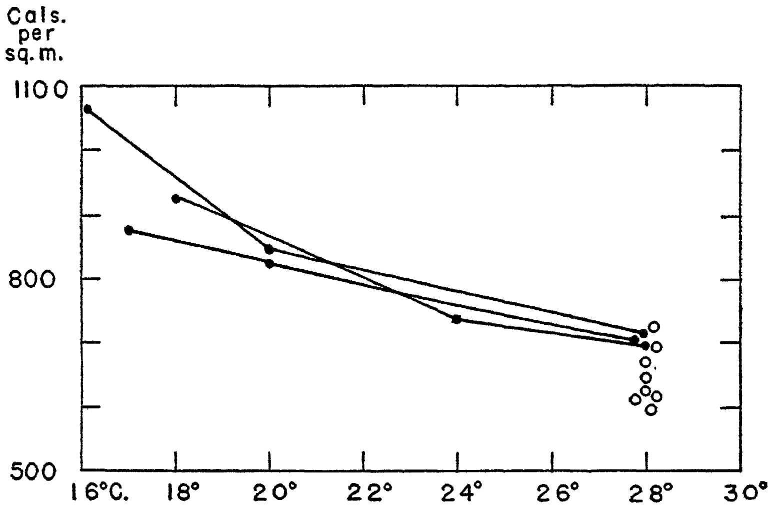

Fig. 1 Influence of environmental temperature upon heat production per square meter of surface area per 24 hours—rabbit 12. The animal had been 24 hours without food at the start of each experiment. Each curve represents the results obtained at successively higher environmental temperatures on the same day, after the rabbit had been living at 17° to 21°C. for more than 3 weeks. The measurements at each temperature did not start until the rabbit had been at the given temperature for about 40 minutes or longer. The hollow circles represent measurements made at 28° after the rabbit had been living 24 hours or more at this temperature. Each plotted value represents the average of several periods of measurement.

2 hours (including about 40 minutes prior to the metabolism measurement). Although these particular curves are based upon the results obtained with one rabbit, they are also representative of those found with seven other rabbits. The metabolism was lowered by the increased temperatures. This

is contrary to the findings of several investigators (Bacq, '29 a, '29 b; Le Breton and Schaeffer, '33; Le Breton, '34 a), who reported thermic neutrality for the rabbit to be as low as 20°C. Measurements at 28°, 30°, and 32°C. on the same day are recorded in table 2. The rabbits had been fasting 20 to 72 hours and had been living at 26° to 33°C. Although there were variations, in general the heat production was essentially constant, the average values at the three temperatures differing from each other by less than 1%. Measurements at 34° and 36° showed increases of 16% and 15% above the level at 32°C. In twenty-three experiments on sixteen

TABLE 2

Heat production of rabbits[1] *measured at different temperatures on the same day*

RABBIT NO.	NUMBER OF EXPERIMENTS	CALORIES PER SQ.M. PER 24 HOURS		
		28°C.	30°C.	32°C.
2	3	542	512	525
4	1	617	586	616
9	3	558	563	562
12	1	649	615	584
13	1	673	669	642
19	1	682	694	702
63	2	851	930	906
Av.		653	653	648

[1] Kept at from 26° to 33°C. for 24 hours before experiment; post-absorptive.

rabbits habituated for 24 hours to 28° and measured both at 28° and at 24° on the same day, the metabolism at 24° averaged 5% higher than that at 28°C.; a few measurements at 26° were at an intermediate level. With three of the sixteen animals the heat values were lower at 24° than at 28°, averaging 3.8% lower. From the data presented, as well as supplementary data, it is concluded that the zone of thermic neutrality for the rabbit is from 28° to 32°C.

With one exception, the open circles in figure 1 at 28° are below the levels of the curves at this temperature. For 24 hours prior to the measurements represented by these open circles the rabbit had been living at 28°, whereas previous to the measurements at the lower temperatures (represented by

the curves) it had been at about 18°C. From the data on a number of rabbits studied under varying conditions as to environmental temperature, we find that if rabbits have been living below thermic neutrality but above 20°C., habituation to thermic neutrality for an additional 24-hour period prior to measurement will permit normal adjustment of the metabolism to the basal level.

Adjustment to laboratory manipulations. As noted by Le Breton ('34 a) and others, the first experiment made on an animal is often significantly high because of the animal's lack of adjustment to laboratory procedures. In the initial experiments on animals studied on 4 or more days the metabolism averaged 6.3% above the average basal level in subsequent experiments; in half of the instances it was 7.5% or more above the average level. The initial experiment on each animal has been omitted from its average value reported in table 3 (p. 484), except for rabbits borrowed from other laboratories, as these latter were accustomed to laboratory handling and as consideration of their initial measurements shows agreement with the average values obtained with other rabbits in numerous experiments.

Time of day. The heat production of five rabbits (3.5 to 4.6 kg.) at thermic neutrality was measured at four different times of day. The animals had been 24 hours without food at the start of each experiment. Although the length of the fast was greater as the experiment progressed, any effect of this factor in obscuring the analysis with regard to time of day was avoided by varying the time of starting the experiments. The results are as follows, the values in italics indicating the initial measurements.

Rabbit no.	*Calories per sq. m. per 24 hours* 9 A.M.	2 P.M.	9 P.M.	2 A.M.
41	*840*	752	756	791
53	683	*716*	730	728
58	*578*	572	592	556
59	783	*682*	739	711
64	656	648	*715*	673
Average	708	674	706	692

There is some variation between the measurements on any one animal but no consistent variation ascribable to time of day. Le Breton ('34 b) also failed to find any consistent diurnal variation in the rabbit's metabolism. Similarly, no characteristic daily rhythm was found in the rabbit's body temperature (Lee, '39).

BASAL METABOLISM

Variability in the individual rabbit's metabolism from day to day. Basal metabolism measurements under the conditions outlined above were made on seventy-four rabbits, of which thirty-four were studied in three or more experiments. Even under presumably uniform conditions, some variability existed between measurements repeated from day to day on the same rabbit. Among eight rabbits studied on 8 or more days each, the mean maximum variation in heat production per square meter of surface area from the average heat production was ±15.3%. The standard deviation of the percentage differences between the individual values for each animal and its average value per square meter was ±7.8% in the ninety-six measurements on these eight animals. For the thirty-four animals measured on 3 or more days the standard deviation of the percentage differences was ±6.9%.

Average basal metabolism of the rabbit species. The average results for our seventy-four rabbits are summarized in table 3. The heat production is expressed in total calories per 24 hours and in calories per square meter of surface area ($S = 0.001w^{2/3}$; $w =$ weight in grams). The metabolism increases with size, the total heat production ranging from 60 calories for a 1.2 kg. rabbit to 335 calories for a 7-kg. rabbit, and the heat production per square meter from 534 to 914 calories. No single value on any of the common bases may be given to represent the heat production of the rabbit species, but the data in table 3 may be averaged according to weight as follows:

Weight group kg.	*Number in group*	*Average weight* kg.	*Calories per 24 hours* Total	Per sq.m.
1	20	1.52	82.6	624
2	13	2.46	119.3	654
3	19	3.57	163.7	699
4	14	4.33	191.1	721
5	7	5.33	232.7	761

A number of investigators have reported heat values for rabbits expressed with reference to surface area but have failed to state the formula used for calculating the area, which prevents intelligent comparison of the results. We here enter a plea for the use of a uniform method of making such calculations. In those instances where comparisons are possible, we find that most of the previously published values (calculated per $0.001w^{2/3}$; w = weight in grams) for the basal heat production of the rabbit are higher than ours. Possibly the explanation is that the measurements were not made under all the prerequisite conditions outlined above. Our values are among the minimum. Other values that agree closely with ours, when weight is taken into consideration, are those reported by Laufberger ('25), Ghoneim ('30), Le Breton and Schaeffer ('33), Le Breton ('34 b), and Smuts ('35), and some of the minimum values of Webster, Clawson and Chesney ('28).

Sex. Comparison of the data with regard to sex shows essentially the same range in percentage variability from the average trend for total heat production referred to weight for each sex, but the twenty-three males have a metabolism 2% higher than that of the females when their average metabolic trends with increasing weights are considered. Hence, a sex difference, which has been found pronouncedly in humans (Gephart and DuBois, '16; Harris and Benedict, '19) and to a less extent in rats (Horst et al., '34 a, '34 b), is suggested by these data on rabbits.

Rectal temperature in relation to metabolism. The normal limits of the rectal temperatures of adult rabbits are from 38.4° to 41.1°C. (Lee, '39). The rectal temperature measure-

TABLE 3

Basal metabolism of rabbits [1]

(Average values)

RABBIT NO., BREED,[2] AND SEX			WEIGHT	NUMBER OF EXPERIMENTS	RECTAL TEMP. [3]	R. Q.	HEAT PRODUCED PER 24 HOURS	
							Total	Per sq.m.[4]
			kg.		*°C.*		*Cals.*	*Cals.*
1	P	♂	1.15	3	39.1	0.72	77.7	706
2	P	♂	1.18	14	39.3	0.72	59.8	534
3		♂	1.18	1		0.73	69.6	621
4		♀	1.22	2		0.73	67.8	592
5	P	♀	1.28	5	38.7	0.72	72.3	613
6		♂	1.29	1		0.75	75.2	632
7		♂	1.31	2		0.73	66.5	558
8	P	♂	1.31	5	39.1	0.70	75.5	629
9	P	♀	1.37	21	39.4	0.72	72.7	591
10		♂	1.43	2		0.73	69.5	550
11			1.49	1	39.3	0.71	78.2	602
12	P	♀	1.52	17	39.6	0.71	82.1	622
13		♀	1.70	2	39.8	0.75	92.2	649
14	E	♂	1.70	1		0.76	91.4	644
15		♀	1.78	1		0.73	83.8	570
16	D	♂	1.81	1		0.72	97.8	656
17		♀	1.86	2	39.2	0.74	86.7	574
18		♀	1.90	2	39.6	0.83	108.0	706
19		♀	1.93	2		0.74	105.9	684
20		♀	1.97	1		0.74	118.3	754
21		♀	2.06	1	39.4	0.71	95.4	589
22	H	♀	2.06	2	38.6	0.71	103.0	638
23		♂	2.17	1	38.6	0.71	101.2	602
24	H	♂	2.23	3	39.0	0.72	91.4	535
25		♂	2.38	1		0.77	140.3	788
26		♀	2.40	1		0.76	112.8	630
27	D	♂	2.42	4	39.0	0.73	128.4	713
28		♀	2.47	1	39.5	0.76	116.1	634
29		♀	2.67	1	39.4	0.78	127.8	666
30		♀	2.69	1	39.2	0.75	112.1	581
31	C	♀	2.79	4	39.8	0.74	143.1	723
32		♀	2.80	1	39.1	0.74	138.4	696
33	J	♂	2.84	1		0.74	141.4	703
34	F	♂	3.11	3	39.6	0.73	118.1	554
35		♀	3.13	1	39.7	0.77	140.8	658
36		♂	3.20	1		0.74	157.8	727
37	Z	♀	3.21	4	39.6	0.74	132.5	608
38	Z	♀	3.30	3	39.2	0.73	149.1	672
39		♀	3.37	1	39.4	0.74	182.8	812
40	Z	♂	3.43	1	39.6	0.74	196.8	867

TABLE 3 (*Continued*)

RABBIT NO., BREED [2] AND SEX			WEIGHT	NUMBER OF EXPERIMENTS	RECTAL TEMP. [3]	R. Q.	HEAT PRODUCED PER 24 HOURS	
							Total	Per sq.m.[4]
			kg.		*°C.*		*Cals.*	*Cals.*
41	Z	♀	3.55	7	39.7	0.76	161.1	691
42	E	♀	3.56	1	39.1	0.74	141.2	606
43		♀	3.57	1	39.1	0.74	121.1	518
44	Z	♀	3.69	4	40.3	0.76	184.8	773
45		♀	3.69	2	39.4	0.74	164.8	691
46		♀	3.77	1		0.76	218.4	902
47	Z	♀	3.81	3	39.6	0.71	160.3	657
48		♀	3.83	1	39.0	0.75	170.8	697
49		♀	3.87	2	39.8	0.73	180.4	734
50	E	♀	3.89	2	39.2	0.74	148.7	601
51	Z	♂	3.97	1		0.71	159.4	635
52	Z	♂	3.97	3	40.2	0.73	221.7	883
53	Z	♀	4.02	8	40.0	0.76	189.6	750
54	Z	♀	4.03	5	39.8	0.74	187.8	742
55	Z	♀	4.04	3	39.8	0.74	182.1	717
56	Z	♀	4.11	4	39.7	0.74	213.9	832
57	Z	♀	4.13	1		0.77	173.8	676
58	Z	♀	4.14	8	39.5	0.76	184.5	715
59	Z	♀	4.21	9	39.8	0.78	211.8	811
60	Z	♀	4.27	4	39.6	0.74	201.2	765
61	Z	♀	4.39	4	39.2	0.74	180.1	672
62	Z	♀	4.42	4	39.7	0.72	157.1	584
63	G	♀	4.55	11	40.3	0.71	233.1	848
64	Z	♀	4.55	6	39.4	0.77	199.1	724
65	Z	♀	4.83	4	39.4	0.73	173.3	606
66	Z	♂	4.87	4	39.3	0.72	188.4	656
67	F	♂	5.16	3	39.5	0.71	239.6	801
68	Z	♀	5.17	8	40.1	0.70	180.2	603
69	F	♂	5.20	3	39.6	0.70	224.2	747
70	Z	♀	5.28	4	39.8	0.77	209.1	690
71	F	♀	5.39	1		0.75	243.2	792
72	F	♀	5.47	1		0.72	256.5	825
73	G	♂	5.67	1		0.71	275.9	868
74	F	♀	7.00	4	40.2	0.74	334.5	914

[1] Measured at 28° to 32°, between twenty-fourth and seventy-second hours of fasting; kept at thermic neutrality for 24 hours or longer before experiment.

[2] The breed is indicated by letters as follows: C = Chinchilla; D = Dutch; E = English; G = Checkered Giant; H = Himalayan; J = Japanese; P = Polish; Z = New Zealand. Where no letter follows the rabbit number, the breed is mixed or unknown.

[3] Average of the temperatures recorded at the end of each metabolism experiment.

[4] $S = 0.001w^{2/3}$; w = weight in grams.

ments accompanying the basal metabolism measurements were always within these limits. The heat production of individual rabbits with differing rectal temperatures within this range was carefully studied, but no consistent tendency was found among individual animals for high body temperature to be correlated with high heat production or vice versa. When the values for the different animals as a whole are considered, a correlation is noted between the heat production per square meter and the rectal temperature. The rectal temperatures were slightly higher and the heat production per square meter averaged appreciably higher at the heavier weights than at the lighter weights. How significant this correlation is, however, cannot be established by our limited data.

Size. The relationship between size and basal metabolism, already briefly discussed in the presentation of the basal data (p. 482), will be considered more in detail in a subsequent report.

SUMMARY

For basal metabolism measurements on normal adult rabbits, animals in good nutritive state with rectal temperatures between 38.4° and 41.1°C. should be used. The measurements can be made at any time of day or night. Absence of muscular activity is necessary. The rabbits should have fasted for from 24 to 72 hours prior to measurement, should have been living at environmental temperatures above 20° but not above 32°, and should have been habituated to the environment of thermic neutrality (28° to 32°C.) for 24 hours or longer prior to the observations at thermic neutrality. The initial experiment on any rabbit not previously accustomed to laboratory procedures should be considered only as an orientation experiment.

The rabbit's basal metabolism was established by 251 experiments on seventy-four adult animals, ranging in weight from 1 to 7 kg. The total heat production ranged from 60 to 335 calories per 24 hours and the heat production per square meter of surface area from 534 to 914 calories, being in general higher the heavier the weight. The males tended to

have a slightly higher metabolism than the females. There was no pronounced correlation between rectal temperature and heat production. The heat values per square meter were lower than most of the values reported in the literature, probably because of strict adherence to basal conditions of measurement.

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SIZE AND BASAL METABOLISM OF THE ADULT RABBIT

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TWO FIGURES

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The relationship of heat production to size among different animal species has frequently been studied, notably by Rubner (1883), Kleiber ('32), Brody ('34), and Benedict ('38), and various attempts have been made to correct for differences in size to bring the results for all animals to a comparable basis. This desirable end has not been fully attained. Even though differences in size may be to some extent ruled out by consideration only of various animal species of relatively the same body weights, there are numerous difficulties in comparing the heat productions of these animal species, such as differences in body configuration, body composition, and body covering. Thus far, the study of the factor of size per se has not been made under the most advantageous conditions. A better opportunity to study this factor is afforded by an animal species that has a large variation in the adult weights of various breeds within the species. The rabbit species is particularly suited for this study. It is possible to obtain rabbits having a sevenfold range in adult weights and yet, in spite of this large weight difference, having a configuration that is relatively unchanged (Castle, '30). Furthermore, the body composition is essentially the same in the large as in the medium and small-sized adult rabbits, as we have found from our analyses of the bodies of rabbits. Hence the difference in weight between the small and the large adult rabbit is not

a difference in the percentage content of body fat. In addition, domestic rabbits of all sizes live under practically the same conditions, having the same daily routine of feeding and activity, which is probably not true of many other animal species having as wide a range in adult weight. Therefore, for the study of the effect of difference in size upon metabolism the rabbit presents as nearly ideal conditions as are obtainable in any species. As the most practical index of size ordinarily available is the body weight, our consideration of size will be made with reference to body weight, and the terms 'body weight' and 'size' will be used synonymously. The discussion will be based upon our basal metabolism study of adult rabbits, the results of which have already been published (Lee, '39), and we shall seek to find some method of expression of the data by which the average trend of the heat production will be related to the weight as a simple function (or linear equation) or by which some direct relationship between the total heat production and the weight can be stated mathematically.

Total heat production referred to body weight. In figure 1 the values for the basal total heat production of our rabbits per 24 hours have been plotted with reference to the body weight. These data are the average results on each of seventy-four adult rabbits. Through the plotted values a curve has been drawn to indicate the general trend of the metabolism between the weights of 1 to 7 kg. Because of the variability in the metabolism of the different rabbits, the total heat production in a few instances is the same for rabbits differing in weight by as much as 1.5 kg. However, on the average, the total heat production between the weight limits of 1 to 7 kg. increases as a straight-line function of the increase in weight. In other words, the amount of increment in total heat production per kilogram of increase in weight above 1 kg. is the same at all weights. The percentage deviations of the measured heat production from the average metabolism as indicated by this curve have been calculated, and in table 1 the sum of the plus and the sum of the minus deviations (column c)

have been recorded for each weight group. For each group the difference between these two sums is small, which means that the straight-line curve has been drawn correctly to represent the average trend of the data throughout the entire weight range. The average total heat productions of the several weight groups, as calculated from the curve in figure 1 and as actually measured, are recorded in columns d and e of table 1. The close agreement between the measured and the calculated metabolism is further proof of the validity of the linear re-

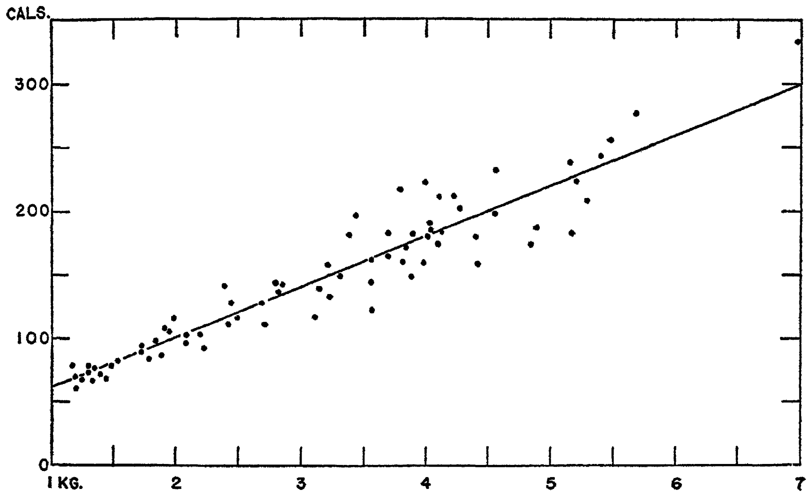

Fig. 1 Basal total heat production per 24 hours referred to body weight of rabbit.

lationship. According to the curve in figure 1 the average total heat production of the adult rabbit can be predicted by the formula $h_{total} = 39.35W + 22.5$, in which h_{total} represents total calories per 24 hours and W, the weight in kilograms. As the total heat production increases as a linear function of the increase in weight, the total heat production is related to weight.

Heat production per kilogram of body weight. If the average values for the total heat production at each kilogram of body weight as indicated by the curve in figure 1 are divided

TABLE 1

Comparison of the rabbit's measured heat production with that calculated on various bases
(Average trend of metabolism referred to weight)

WEIGHT (GROUP AND AVERAGE)	NUMBER OF RABBITS	TOTAL HEAT PRODUCTION PER 24 HOURS				PARABOLIC CURVE		HEAT PRODUCTION PER SQ.M. PER 24 HOURS				HEAT PRODUCTION PER $W^{0.73}$ PER 24 HOURS			
		Sum of per cent deviations of measured from calculated[1] heat	Average		Standard deviation	Sum of per cent deviations of measured from calculated[2] heat	Calculated heat[2]	Sum of per cent deviations of measured from calculated[3] heat	Average		Standard deviation	Sum of per cent deviations of measured from calculated[4] heat	Average		Standard deviation
			Calculated[1]	Measured					Calculated[3]	Measured			Calculated[4]	Measured	
kg. (a)	(b)	(c)	*Cals.* (d)	*Cals.* (e)	(f)	(g)	(h)	(i)	*Cals.* (j)	*Cals.* (k)	(l)	(m)	*Cals.* (n)	*Cals.* (o)	(p)
1.0 to 1.99 1.52	20	+ 66.8 − 67.1	82.3	82.6	± 8.4	+ 68.7 − 62.0	82.1	+ 65.1 − 66.3	625	624	± 8.3	+ 70.4 − 62.4	60.6	60.8	± 8.3
2.0 to 2.99 2.46	13	+ 47.8 − 51.0	119.3	119.3	± 9.6	+ 37.3 − 64.8	121.6	+ 44.8 − 54.9	658	654	± 9.6	+ 43.8 − 56.8	62.4	61.8	± 9.6
3.0 to 3.99 3.57	19	+114.6 −110.9	163.2	163.7	±14.8	+107.3 −121.2	164.6	+113.8 −113.2	699	699	±14.8	+113.0 −112.5	64.5	64.5	±14.8
4.0 to 4.99 4.33	14	+ 58.8 − 66.0	192.7	191.1	±11.1	+ 59.1 − 63.0	192.2	+ 58.4 − 66.1	726	721	±11.0	+ 60.9 − 63.0	65.9	65.8	±11.0
5.0 to 5.99 5.33	7	+ 30.2 − 30.7	232.4	232.7	±10.4	+ 39.8 − 26.3	227.9	+ 29.4 − 30.7	762	761	±10.3	+ 34.2 − 28.3	67.8	68.4	±10.6
7.0	1	+ 12.2	298.0	334.5		+ 17.8	283.9	+ 11.2	822	914		+ 13.8	71.0	80.8	
S. D. for all 74 rabbits					±11.3						±11.2				±11.3

[1] Calculated from curve in figure 1.

[2] Calculated from equation: total 24-hour heat production $= 58.7W^{0.81}$ in which W represents weight in kilograms. See column e for measured total heat production.

[3] Calculated from curve in figure 2.

[4] Calculated from curve giving equation: heat production per $W^{0.73} = 1.9W + 57.7$.

by the respective weights, the resulting values for the heat production per kilogram per 24 hours are as follows:

Weight in kilograms	1	2	3	4	5	6	7
Calories per kilogram per 24 hours	61.9	50.6	46.9	45.0	43.9	43.1	42.6

Hence, as is generally recognized, a single value for heat production per kilogram of weight will not suffice to express the metabolism of rabbits of different weights, for the heat production per kilogram decreases rapidly at the lighter weights and less rapidly at the heavier weights. The value

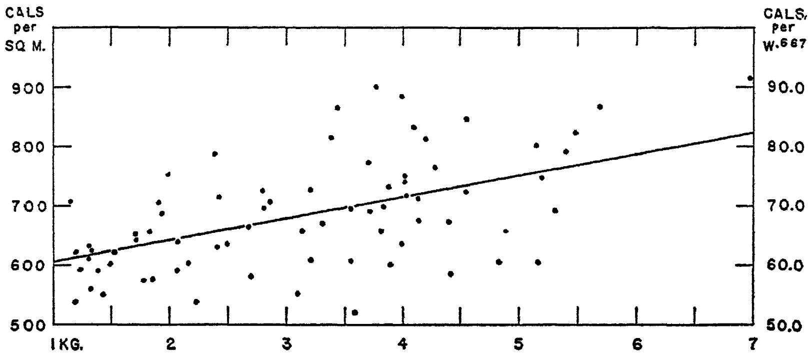

Fig. 2 Basal heat production per square meter of surface area per 24 hours referred to body weight of rabbit. The left-hand scale of ordinates represents the calories per square meter of surface area, obtained by dividing the total 24-hour heat production by $0.001w^{2/3}$, in which w represents the weight in grams. The right-hand scale of ordinates represents the calories per $W^{0.667}$, in which W is weight in kilograms.

at 7 kg. is 31% lower than that at 1 kg., and even between 1 and 3 kg., the weights most frequently found in the literature, the values decrease 24%. Therefore the change in the heat production per kilogram is not a simple function of the increment in weight, i.e., the relationship cannot be expressed as a linear equation.

Heat production per unit of surface area. In figure 2 the values for the average 24-hour heat production per square meter of surface area (left-hand scale of ordinates) have been plotted with reference to weight. The surface area was ob-

tained by calculation, using the Meeh formula $S = \frac{K \times W^{2/3}}{10{,}000}$, where S is the surface area in square meters, W is the weight in grams, and K equals 10. Correction for the surface areas represented by rabbit's ears, which has been stressed by Rubner ('02), did not have to be made in our calculations, because our rabbits all had the same relative configuration and the present analysis is confined to the rabbit species alone. The curve in figure 2 indicates the average trend of the data. Although figures 1 and 2 give the impression that the scatter of the plotted heat values per square meter is much greater than the scatter of the total heat values, actually the average of the percentage deviations of measured heat production per square meter from that indicated by the curve is only slightly greater than the average of the percentage deviations in the case of the total heat production. The sums of the plus and the minus deviations for each weight group, recorded in column i of table 1, show that the straight-line curve in figure 2 represents the average trend of the data well. Comparison of the averages of the measured and the calculated values per square meter of surface area for each weight group (columns k and j) supports this thesis. Hence the relationship between the heat production per square meter of body surface and the body weight can be expressed by an equation of the first degree (a linear equation). In other words, the rabbit's basal heat production per square meter can be predicted from the formula $h_{sq.m.} = 36W + 570$, in which $h_{sq.m.}$ represents calories per square meter of surface area (calculated according to the formula given above) per 24 hours and W, weight in kilograms.

Heat production referred to powers of the weight. In the attempt to clear up the relationship between size and metabolism, Krogh ('16) has proposed expressing the heat production as a function of W^n rather than per square meter or any other unit of surface area, and he recommends that for warm-blooded animals n be taken, at least provisionally, as 2/3. The 0.73 power has been suggested by Brody ('34), Kleiber ('32), and the Committee on Animal Nutrition of the National

Research Council ('35). The individual total heat values for our rabbits were divided by several different powers of their body weights (in all cases expressed as kilograms) from 0.50 to 1.00 and plotted with reference to weight on each basis. For all the powers between 0.61 and 0.73, we consider that the general trend of the data is best indicated by a straight line, but for powers above and below these the median deviates from a straight line. Between the weight limits of 1 to 7 kg., the values for the heat production per $W^{0.667}$ are almost exactly one-tenth of the values per square meter of calculated surface area. Hence figure 2 (using the right-hand scale) shows the trend of the data per $W^{0.667}$ as well as per unit of surface area. For all powers of W between 0.61 and 0.73, the percentage deviations of the measured values from the medians are of essentially the same magnitude in the respective weight groups. In table 1, column m, the sums of the plus and the minus deviations for each weight group are given for the heat production per $W^{0.73}$, as an example. Since the 0.73 power is one of the limits of the powers showing a straight-line relationship between heat production per W^n and weight, the plus and the minus deviations for the several weight groups do not balance each other so well as is the case when the heat production is expressed with reference to other powers of the weight within the limits of the powers 0.61 and 0.73. The sums of the plus and the minus percentage deviations as recorded in column m of table 1 and the agreement of the calculated values with the measured values in columns n and o show that the straight-line curve correctly indicates for each weight group the average heat production per $W^{0.73}$. As this is also true for the other powers of W from 0.61 to 0.73, the heat production per W^n for values of n between these limits is related to weight by an equation of the first degree.

Total heat production as a parabolic function of the weight. As, theoretically, the heat production at zero weight would be zero, and as the basal total heat production of rabbits weighing from 1 to 7 kg. increases with the increase in weight, a

curve of the parabolic type passing through the origin or zero point would seem logically to apply to these data. The parabolic curve best fitting these data is indicated by the equation $h_{total} = 58.7W^{0.81}$ in which h_{total} represents the total 24-hour heat production and W, the weight in kilograms. In column g of table 1 are given the sums of the plus and the minus percentage deviations of the measured heat production from the metabolism as indicated by this parabolic curve. The difference between these two sums for each weight group shows that this curve does not fit the data so well as the straight-line curves. Other parabolic curves have been proposed for relating heat production to weight. Brody has proposed the formula $h_{total} = 70.5W^{0.734}$ and Kleiber, $h_{total} = 72W^{0.75}$. Neither of these conforms well to our data for the rabbit. Although at 7 kg. the heat values as predicted by the formulas of Brody and Kleiber are about the same as the average trend of the total heat production of our rabbits, at 1 kg. the values predicted by these formulas are 14% and 16%, respectively, above the average trend of our data at this point. However, the formulas of Brody and Kleiber are based on differences in size over a wide weight range, including many different animal species.

Comparison of the valid simple relationships of heat production to size. The rabbit's metabolism has been shown to have a linear relationship to weight when expressed as the total heat production, the heat production per square meter of surface area, and the heat production per W^n for values of n from 0.61 to 0.73. In all instances, however, the straight-line curves representing this relationship have an upward slope and are not horizontal. Hence, we have found no means of equalizing differences in the sizes of rabbits whereby a single value will accurately express, on the average, the basal metabolism of all rabbits. Whether such a constant relationship would be shown if the surface areas of our rabbits had been actually measured instead of calculated is a question that cannot be answered by our data. The measurement of the surface areas would probably result in a more exact factor for cal-

culating the area but probably would not eliminate the differences in heat value per square meter unless it happened that the factors varied according to increasing weights. The next question is, which straight-line curve will best express the rabbit's basal metabolism. When the sums of the plus and the minus deviations of the measured values from those calculated (for the same weight group) from each of the several straight-line curves are compared (recorded in columns c, i, and m of table 1 for three of the curves), the closest balances between the plus and the minus values in the several weight groups are those noted in column c for the total heat production. This signifies that the curve for total heat production expresses most accurately the relationship between the rabbit's heat production and its weight. In columns f, l and p of table 1 the standard deviations of the measured from the calculated metabolism, expressed as percentages, have been recorded for each weight group on the bases of total heat production, heat production per square meter of surface area, and heat production per $W^{0.73}$. The standard deviations are practically alike on all three bases for each weight group. For all seventy-four rabbits the standard deviations are also essentially the same on all three bases (see last horizontal line of values in table 1). Of all the relationships studied, although several were nearly as good, the best and likewise the simplest relationship found was the linear relationship between total heat production and body weight. The exact expression of this relationship is $h_{total} = 39.35W + 22.5$. For practical purposes, however, the equation $h_{total} = 40W + 20$ will serve as a more convenient method of calculation. With other animal species straight-line relationships between total heat production and increase in weight within the individual species have also been found, but the rates of increase in total heat production with increase in weight are not alike for all species (Benedict, '38).

Heat production of the rabbit compared with heat productions of other animals of the same weight. Comparison of the heat production of the rabbit with the heat productions of

other warm-blooded animal species of approximately the same weight is of general physiological interest. The comparison is best based on studies made by the Nutrition Laboratory, as, from the standpoint of technic, the conditions of measurement of the different animal species were thus the most uniform. The results obtained with the various animal species studied by the Nutrition Laboratory and by one or two other investigators have been summarized by Benedict ('38). The data for the rabbit given by Benedict include, in part, the basal values reported by Lee ('39) and, in part, the values reported by a few other investigators. Hence, Benedict's average for the rabbit is slightly different from the value given in this

TABLE 2

Comparison of total heat production of warm-blooded animals of like weight[1]

WEIGHT IN KILOGRAMS	CALORIES (AVERAGE) PER 24 HOURS							
	Rabbit	Wild birds	Marmot	Macaque	Goose	Hen	Cock	Cat
1	61.9	87.5						
2	101.2	130.0	62.5			112.0		100.0
3	140.6	172.5	81.0	167.0	173.5	146.0	157.0	152.5
4	179.9	216.0	100.0	199.0	222.5			
5	219.3	259.0		232.0	272.5			

[1] Data for rabbit derived from curve in figure 1; data for other animals derived from curves in figure 29 in the monograph of Benedict ('38).

present study. For the best comparison of the rabbit's metabolism with that of other warm-blooded animals of the same weight, the heat productions at different weights within the range from 1 to 5 kg. have been recorded in table 2 on the basis of total heat production. The values for all the animals except the rabbit have been obtained from the curves shown by Benedict in figure 29 of his monograph. For the rabbit, the values have been derived from figure 1 of this present paper. It is unfortunate that so few values for mammals are available at this weight range. One of the mammals, the marmot, is a hibernating animal, and its total heat production, even under normal, awake conditions, is much lower than that of other warm-blooded animals of like weight. Of the eight animals

represented in table 2, four are birds. It is generally recognized that birds have a higher metabolic level than mammals. Therefore, the comparison in table 2 is far from ideal. If it is legitimate to draw any conclusion from these few values, it can be stated that the rabbit has a metabolism slightly below the average levels of the other animals, except for the marmot.

On the basis of the average heat production per square meter of surface area (using for the rabbit figure 2 of this paper and for other warm-blooded animals of like weight Benedict's general curve for animals weighing from 20 gm. to 4,000 kg., given in figure 44 of his monograph), we find the following comparison:

Body weight, kg.	1	2	3	4	5	6
Calories per square meter						
Benedict's average	680	708	720	742	753	762
Rabbit	606	642	678	714	750	786

Here it is apparent that the rabbit's metabolism is below the general average at the lower weights but agrees with the general average at 5 kg. No attempt has been made in the above comparison to refine the calculations for the rabbit to correct for its relatively greater ear area as compared with that of other animal species, for we are concerned here not with the relationship between heat loss and surface area but with the relationship between heat production and size (weight).

SUMMARY

There is no known method of equalizing differences in the sizes of rabbits, which have a sevenfold range in adult weights, whereby a single value will accurately express the average basal metabolism of all rabbits. Within the rabbit species the relationship between basal metabolism and size is best expressed by referring the total heat production to the weight. For each kilogram increase in weight above 1 kg. up to 7 kg., the total heat production increases at a constant rate and can be predicted by the equation $h_{total} = 40W + 20$, in which h_{total} represents kilogram-calories per 24 hours and W, the weight in kilograms. Other simple linear relationships

between metabolism and size are found when the heat production is expressed per square meter of surface area and per W^n for values of n between 0.61 and 0.73, inclusive. The total heat production of the rabbit is slightly below the average metabolism of other warm-blooded animals of like weight, with the exception of the marmot. At 5 kg., its average heat production per square meter of surface area agrees with the general average for other warm-blooded animals, but at the lower weights it is below the general average.

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RESTORATION OF FERTILITY IN SUCCESSIVELY OLDER E-LOW FEMALE RATS [1]

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Several years ago it was noted in this laboratory that the usual 'curative' dose of wheat germ oil did not invariably lead to the birth of living young when administered later than the first half year of life to E-low female rats of proved sterility. This led to standardization of the test animal as a female under 6 months of age whose trial resorption gestation was begun at 60 days.

In the study herein presented, nearly 300 rats, ranging in age from 3 to 18 months, have been given graded doses of wheat germ oil. All rats were reared on the standard E-low diet 427 [2] and were bred for their trial gestation at the first

[1] Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, from Merck and Company, Inc., and the Rockefeller Foundation, New York. Assistance was rendered by the Works Progress Administration, Project no. 10482-A5. The following materials were generously contributed: cod liver oil by E. R. Squibb and Sons, brewers' yeast by The Vitamin Food Company of New York, and wheat germ from which the oil was prepared by General Mills, Inc.

[2] Diet 427:

	%
Casein (commercial)	27
Cornstarch (cooked)	35
Lard	22
Cod liver oil	2
Yeast	10
Salt mixture no. 185	4

(The mixed diet without the cod liver oil was allowed to stand for 2 weeks at room temperature to permit the rancid substances of the lard to destroy incipient traces of vitamin E. The cod liver oil was added just before feeding.)

pro-estrus after the sixtieth day of age. The wheat germ oil was administered in a single dose by stomach tube on the day vaginal spermatozoa were encountered in the case of the groups receiving the lower levels of oil. In the group receiving 4 gm. of wheat germ oil, a 2-gm. dose of the oil was given on the first day and the remaining 2 gm. on the following day. In the case of the group on the 10-gm. level, five doses of 2 gm. each were given on alternate days.

Groups were bred at monthly intervals but in table 1, age groups which showed similar responses are combined. The

TABLE 1

Capacity of wheat germ oil to restore fertility to successively older female rats of proved sterility

WHEAT GERM OIL	AGE IN MONTHS	NUMBER OF COPULATIONS	PLACENTAL SIGN		NUMBER OF LITTERS	PER CENT LITTERING AFTER SHOWING PLACENTAL SIGN	AVERAGE NUMBER LIVING YOUNG PER LITTER
gm.			*Number*	*%*			
0.5	3–6	21	21	100	21	100	7.3
0.5	7–9	34	28	82	15	54	4.3
0.5	10–11	19	16	84	1	6	5.0
1.0	7–9	31	22	71	13	59	6.5
1.0	10–11	30	21	70	1	5	3.0
2.0	8–10	32	23	72	10	43	5.4
2.0	11–12	30	16	53	1	6	3.0
3.0	9–12	30	20	67	5	25	2.4
4.0	11–12	32	20	63	6	30	3.0
10.0	15–18	24	8	33	0	0	0.0

table shows that during the first half year of life, a single dose of 0.5 gm. of wheat germ oil invariably led to the birth of living young. An inferior outcome when 0.25 gm. wheat germ oil was fed has led us to designate 0.5 gm. as the minimal effective single dose (MED) of wheat germ oil for the restoration of fertility. The same dose, when administered from the seventh to the end of the ninth month of life, led to the birth of living young in only half the cases, and when given from the tenth to the eleventh month, was almost entirely inefficacious. A considerable elevation of wheat germ oil dosage

(two to four times) in the period elapsing from the seventh to the eleventh month does not substantially increase the instances of the birth of living young. Yet pronounced elevation of dosage does have an effect. From the eleventh to the twelfth month (a period, as has been mentioned, of virtually complete ineffectiveness of the MED of wheat germ oil) an eightfold elevation of the dose (4 gm.) led to the birth of living young in approximately a third of the cases (although the litter size remained subnormal).[3] From the fifteenth to the eighteenth month of life the administration of as much as 10 gm. of wheat germ oil did not lead to the birth of living young.

Until the end of the first half year of life, interference with implantation was not observed. In a total of 1211 test animals 3 to 6 months of age and of proved sterility, 94.7% have shown the placental sign following the next positive mating. This figure happens to be actually somewhat above the average for our stock colony (93%). This is not in agreement with the reports of Bacharach et al. ('37, '38) who state that they have found a clearly subnormal proportion of implantations in animals which had undergone a resorption gestation. Nevertheless, a comparison of the instances of copulation and the implantations following shows that there is evidence of increasing failure in implantation as age progresses. Established occlusion of the oviducts from infection explained about

[3] The possibility of increasing the number of instances of the birth of living young by fourfold dose increase of wheat germ oil was demonstrated by another group of twenty-two females on the E-low regimen but allowed to attain the ninth month of life without a trial gestation. Half of these received 1 gm., half of them 4 gm., of wheat germ oil on the inauguration of a test gestation. The results are subjoined. Here also the litter size was not improved by quadrupling the dose.

WHEAT GERM OIL	AGE IN MONTHS	NUMBER OF COPULATIONS	PLACENTAL SIGN		NUMBER OF LITTERS	PER CENT LITTERING AFTER SHOWING PLACENTAL SIGN	AVERAGE NUMBER LIVING YOUNG PER LITTER
gm.			*Number*	*%*			
1.0	9–10	11	10	91	4	40	4.5
4.0	9–10	11	10	91	7	70	4.3

half of the implantation failures, but we are not yet ready to state to what extent actual failure of the implantation mechanism accounts for the remaining half of such cases. In animals 15 to 18 months old no implantation sites were ever encountered, although uterine bleeding had been observed.

At autopsies, the uteri were invariably dark brown in color and were usually stiff and hard. Martin and Moore ('36, '38) found that rats (whether virgins or with the history of a single resorption gestation) when maintained for long periods on a vitamin E-low diet, developed a brown uterus which could not be restored to normal color either by treatment with a vitamin E concentrate or by shift to a diet consisting of natural food. Barrie ('38) found that the uterine pigmentation progressed with age. After 4 months on the diet there was a slight increase in the size of the uterus and definite, although slight, discoloration; after 5 months the discoloration and enlargement [4] were more marked and the condition was markedly expressed after 6 months. A resorption accelerated the appearance of the pigmentation. Barrie stated that the vitamin E requirement for restoring fertility increased as these changes advanced and postulated that pregnancy would be impossible if the uterine changes had progressed to an advanced stage.

We have given some attention to the phenomenon of uterine pigmentation. Twenty-seven E-low rats approximately 6 months of age were divided into three groups. Group 1 was maintained on the vitamin E-low diet throughout the experiment. Group 2 received 4 drops of wheat germ oil in addition to the vitamin E-low diet. Group 3 received 4 drops of wheat germ oil and were bred at the beginning of the experiment. All individuals were sacrificed at the end of 20 days. In agreement with the findings of Martin and Moore ('36, '38), the uteri were as darkly pigmented after the administration of wheat germ oil as in the control group, if the animals were

[4] The enlargement is not invariable. The average weight of the uteri and oviducts of fourteen of our E-low females 19 to 20 months old, with a history of two pregnancies, was 0.78 gm.; for eight natural-food virgin animals of the same age, the average figure was 1.31 gm.

not bred, but in the case of pregnant animals given wheat germ oil (in all cases living fetuses were found) the uterus was normal in color.[5]

In the present communication, we have contented ourselves with statements of fact and do not venture an explanation of the increasing reproductive disability of increasingly older vitamin E-low females. The need for elevated dosage with E shown by older E-low females can, we feel, hardly be taken to indicate that the embryos require more E with advancing age of the mother, but is preferably interpreted as due to: (1) increasing incapacity of the placental mechanism to deliver adequate vitamin E to the embryos, and (2) final failure of the implantation mechanism.

SUMMARY

The reproductive performance of successively older vitamin E-low female rats permits us to recognize three periods:

1. From the age of 3 to 6 months, at which time the response to a single dose of 0.5 gm. of wheat germ oil invariably leads to the birth of normal sized litters of living young. Only this period can be recommended for assay studies.

2. From the seventh to the twelfth month, at which time the response is variable; in the earlier part of this period only half the animals give birth to living young with this dosage (0.5 gm.) and these are undersized litters; moderate elevation of the dosage does not greatly change the result. Toward the eleventh month practically no animals respond to 0.5 gm. but even in this period great elevation of dosage shows a definite effect, for almost a third of such animals respond to 4.0 gm. of wheat germ oil, with living young but small litters.

3. After the fifteenth month of life no response can be secured with a twentyfold increase of dosage.

[5] Barrie ('38) observed a normal uterus in one instance of a female that had cast young.

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A COMPARISON OF CEREAL AND NON-CEREAL DIETS IN THE PRODUCTION OF RICKETS

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In a series of articles Mellanby[1] has presented evidence indicating that cereal grains contain a specific anti-calcifying factor. This evidence was based principally upon the observations (1) that there is a considerable variation in the rachitogenic properties of different cereals, (2) this difference could not be associated with either the total or relative amounts of calcium and phosphorus in the cereals studied and (3) the severity of the rickets produced was considerably decreased by a preliminary treatment of the grains with hydrochloric acid. Mellanby found that oatmeal produced more severe rickets than any of the other cereals investigated. Thomas and Steenbock ('36) in repeating some of the experiments of Mellanby, especially those of Green and Mellanby ('28) did not find a marked difference in the degree of rickets produced by corn, rolled oats, wheat and rice. Corn appeared to have slightly less calcifying action than the others. In an earlier publication Steenbock, Black and Thomas ('30) had observed that the equalization of the phosphorus content of various cereals by the addition of inorganic phosphates did not make the cereals of equal value in calcification. These authors suggested that all of the phosphorus from the various cereals may not be utilized to the same

[1] For references to most of the articles by Mellanby see the papers by Bruce and Callow ('34), and Green and Mellanby ('28).

degree. A considerable portion of the phosphorus in cereals is in the form of inositolphosphate which is incompletely hydrolyzed in the intestinal tract. Following the suggestion of Steenbock and associates, Bruce and Callow ('34) found that the addition of sodium inositolphosphate to a high calcium-low phosphorus rachitogenic ration was less effective in increasing calcification than an equal amount of phosphorus as disodium phosphate. Lowe and Steenbock ('36) later reported that the presence of liberal amounts of calcium carbonate further decreases the hydrolysis and thus the availability of inositolphosphate. Lowe, Steenbock and Krieger ('39) have recently reported that phytin phosphorus is less effective in increasing calcification in the chick than is an equal amount of inorganic phosphorus.

In all of these experiments cereals and cereal products have been used as the greater part of the diets studied and the conclusions have been based principally upon the comparison of one cereal with another. In an attempt to control the calcium, phosphorus and vitamin D content of the cereal diets other essentials frequently have been overlooked and inadequately supplied. Furthermore the grains themselves have been relied upon to furnish some of the necessary factors. Consequently, when one grain has been substituted for another it is possible that the diets being compared have differed in respect to factors other than those directly concerned with calcification. By using a synthetic diet and supplying these factors as individually purified substances the danger of any deficiencies in this respect could be eliminated. Also the effect of a variation in these factors among diets containing different cereals could be reduced to a minimum. The basal diet of Green and Mellanby ('28) was composed partially of purified ingredients. However, casein was used as the protein which precludes the possibility of having a diet low in phosphorus. In addition they supplied vitamin A by adding rather large quantities of dried cabbage which contains various dietary essentials including small and inconstant amounts of vitamin D. Furthermore, these authors did not compare diets con-

taining cereals with non-cereal diets which is possible when a diet based on individual components is employed.

In a recent report by the present author (Jones, '39) several practical, inexpensive and easily prepared rachitogenic rations composed of purified food materials were described. It was pointed out at that time, since severe rickets in the rat can be produced on purified diets it is improbable that cereals owe their rachitogenic properties to the presence of any specific anti-calcifying factor. In the present paper are reported the results of more detailed studies in which three cereals have been compared with each other and simultaneously with non-cereal diets in their ability to promote calcification in the rat.

EXPERIMENTAL PART

The rats used in these studies were placed on the experimental diet at approximately 25 days of age. They were kept in individual cages and except in one series of experiments daily food consumption of each animal was followed. After 21 days the animals were bled under ether anesthesia. The sera were analyzed for calcium by the method of Clark and Collip ('25) and for phosphorus on the calcium-free filtrate by the method of Gunther and Greenburg ('29). The right femurs were removed for the determination of the percentage of ash and the wrist bones for examination by the line test technic. The ash was determined on the dry, fat-free bone.

Several series of experiments were carried out. In each series five different diets were studied simultaneously with representative litter mate rats on each diet. Grains formed the principal part of three of the diets while the other two were composed primarily of purified materials. In the first series the basal diet consisted of alcoholic extracted fibrin 18%, yeast 5%, salt no. 5 (Jones, '39) 5.3%, agar 2% and carotene solution 0.1%. The diets were completed by adding respectively rolled oats, wheat, yellow corn, dextrinized cornstarch or sucrose to make 100%. The latter two diets are the same as rations no. 570 and no. 573 previously described

(Jones, '39). The particular grains were chosen because they were employed in some of the experiments referred to above and because of their widespread use in human and animal nutrition. Dextrinized cornstarch was used in order to include a comparatively pure cereal product. The ration containing sucrose was free of either cereals or their products. The total phosphorus content of these diets expressed in per cent is as follows: rolled oats 0.36, wheat 0.31, corn 0.26, dextrin 0.09 and sucrose 0.09. Phytin phosphorus was determined on the first three of these diets by the method of Harris and Mosher ('34) with the following results: oats diet—0.26%, wheat diet—0.22% and the corn diet 0.18%. The diets were all comparable in respect to calcium as 3% calcium carbonate was added to each. The results of this series as well as the following series of experiments are summarized in table 1.

A significant portion of the phosphorus in the above diets was present in the yeast. In the next series of experiments (series II) the phosphorus content of the grain diets was decreased by omitting the yeast which reduced the phosphorus in each case by about 0.05%. Grains do not contain sufficient quantities of all of the vitamin B factors for optimum growth in the rat in long-time experiments. However, it is generally considered that in experiments of short duration no marked deficiencies develop when any of the common cereal grains form the greater part of the diet. In the following experiments this apparently was true because growth on the grain diets was as good as on the non-cereal diets. The latter contained 5% yeast. The agar was also omitted from the grain diets of this and the subsequent series. This same experiment was repeated but the femurs were not analyzed for ash. Consequently, the results of the second experiment on these same diets are not included in the table, but, aside from the bone ash, they agreed with those presented in table 1 (series II).

Green and Mellanby emphasized the necessity of having a diet low in calcium in order to demonstrate the presence of the anticalcifying factor. In the next series (III) the same diets as in series II were fed with the exception that salt no. 10

at a level of 3.3% replaced salt no. 5. Salt mixture no. 10 is composed of the following ingredients expressed in relative parts by weight: NaCl 1, $CaCO_3$ 1, KCl 1, $MgSO_4$ (an-

TABLE 1

The comparative rachitogenic action of various cereal and non-cereal diets

DIET	NUMBER OF ANIMALS	DAILY FOOD CONSUMPTION [1]	GAINS IN WEIGHT [1]	SERUM [2] Calcium	SERUM [2] Phosphorus	FEMUR ASH [1]	
		gm.	gm.	mg./100 cc.	mg./100 cc.	mg.	%
			Series I				
Oats	6	7.8	40.3	13.1 [3]	4.3 [3]	42.2	39.6
Wheat	6	8.3	37.4	14.1 [3]	4.3 [3]	28.5	33.2
Corn	6	7.9	28.8	11.9 [3]	2.8 [3]	17.5	23.1
Dextrin	5	5.5	20.0	12.7 [3]	2.1 [3]	13.8	20.2
Sucrose	5	5.8	20.8	12.8 [3]	2.7 [3]	19.2	26.1
			Series II				
Oats	3	6.6	27.0	13.0	3.2	25.0	31.5
Wheat	4	7.2	26.2	12.7	3.3	28.6	31.0
Corn	3	6.4	15.3	12.5	2.8	16.2	21.0
Dextrin	3	6.8	26.7	12.0	2.1	19.5	22.8
Sucrose	3	7.1	24.0	12.5	2.2	21.9	24.6
			Series III				
Oats	3	7.4	48.3	11.7	7.6	61.1	43.6
Wheat	3	8.7	48.0	12.1	5.9	53.5	41.5
Corn	3	7.8	36.3	11.8	4.4	35.3	34.7
Dextrin	3	7.9	33.3	13.2	3.7	25.2	25.8
Sucrose	3	6.8	27.0	12.7	3.9	18.7	23.2
			Series IV				
Oats	3	5.7	21.3	5.8	9.3	34.1	41.2
Wheat	3	6.9	21.3	5.4	9.1	30.4	37.8
Corn	3	6.8	27.3	5.8	8.3	34.0	38.6
Dextrin	3	8.2	37.3	8.9	4.5	27.5	33.1
Sucrose	3	6.5	21.0	6.2	5.5	22.7	30.6
			Series V				
Oats	3		7.3	5.6	11.1	31.3	37.9
Wheat	3		8.7	7.3	12.7	27.7	35.2
Corn	3		8.6	6.1	10.8	28.6	35.1
Dextrin	4		15.0	5.2	23.0	28.3	35.0
Sucrose	4		10.2	lost	lost	29.9	36.8

[1] Average of all animals on each diet.

[2] Analyses on pooled samples.

[3] Average of two pooled samples.

hydrous) 0.2, ferric citrate 0.1 and KI 0.004. Thus the diets used in series III differed from those in series II only in their content of calcium carbonate which was reduced from 3% to 1%.

In series IV no calcium was added; otherwise the diets were the same as in series II and III. The calcium content of these diets in per cent was as follows: rolled oats 0.08, wheat 0.08, corn 0.05, dextrin 0.06 and sucrose 0.06.

As a final series (V) the same diets as those of series IV were used except that phosphorus was added to each. This was done by feeding at a 4% level the Steenbock-Nelson salt no. 40 ('23) modified by omitting the calcium salts but keeping the phosphorus at the original level by the addition of disodium hydrogen phosphate. The amount of phosphorus added to each diet was approximately 0.5%. Food consumption records were not obtained in this series of experiments, but it was obvious that all the animals ate considerably less than in any of the other experiments.

DISCUSSION OF RESULTS

As seen from the results presented in table 1 there is a considerable difference in the degree of calcification produced on the various diets. In the first two series the diets were high in calcium and comparatively low in phosphorus. The cereal diets, however, contained considerably more total phosphorus than the non-cereal diets with the oats and wheat having a higher content than the corn. The degree of calcification, as judged by the bone ash and the concentration of inorganic phosphate in the serum, were considerably greater with the rolled oats and wheat diets than with the corn diet. In the latter case the results obtained were very similar to those on the non-cereal rations in which the total phosphorus was approximately one-third that of the corn. In the first series the gains in weight on the corn were intermediate between the two non-cereal diets and those containing either rolled oats or wheat. The consumption of the corn diet, however, was equal to that of the oats or wheat, whereas the food

intake on the non-cereal diets was somewhat less. As seen from the table this difference in food consumption between the cereal and non-cereal diets was not observed in the other experiments and thus cannot be considered as significant. In series II weight gains on the corn diet were the least of any of the groups, but with only three animals in each group not much importance can be attached to these differences. Food consumption was nearly the same for all groups in this series.

When the calcium carbonate was reduced to 1% as in series III the grain diets all produced considerably more calcification than either of the synthetic diets. In this case, with respect to their ability to produce calcification, the corn ration is intermediate to those of the other two grains and the non-cereal diets. The gains in weight were also less on the corn diet than on the other cereals although, as in the previous experiments, food consumption was about the same.

When no calcium was added as in series IV, the diets became essentially low calcium diets and the limiting factor for calcification was probably calcium and not phosphorus. As the calcium contents of all these diets were nearly equal, the degree of calcification expected would also be about the same. As the data show, this is true especially for the grain diets. The two non-cereal diets were very low in phosphorus and it is possible that in these cases phosphorus was still a limiting factor. The rather low levels of serum phosphate of the animals of these groups as compared with those on the grain diets are additional evidence that this was the case.

In series V sufficient phosphorus was added to all diets to assure an excess of this element. A deficiency of calcium was definitely the factor limiting calcification in each of these rations. The deficiency was of nearly the same degree in all groups and, as the data show, there was no apparent difference in calcification. Several of these animals showed definite tetany during the experiment which probably accounts for the very high phosphorus in the serum of the animals on the dextrin diet. Although this determination was not checked with a duplicate, we have observed at various times extremely

high levels of serum inorganic phosphorus during attacks of tetany.

The results of the examination of the wrist bones by the line test technic were in general in accord with the findings in other respects. Almost without exception the uncalcified areas at the metaphyses were wider and more regular on the synthetic diets than on the cereal diets. This was especially true of series III, although the difference was also very definite in series IV. In the latter series the metaphyses of the animals on the grain diets appeared practically normal but were considerably widened on the non-cereal diets. In the last series of experiments there was no widening of the metaphyses. As judged by this criterion in series I and II, corn was markedly more rachitogenic than either of the other cereals and wheat was slightly more rachitogenic than the rolled oats.

The above data are in accord with the hypothesis that the calcifying power of cereals is dependent primarily upon their contents of calcium and available phosphorus. Thus in the first two series of experiments the phosphorus was the limiting factor with corn containing less than either rolled oats or wheat. In series III, by reducing the amount of calcium carbonate a larger proportion of the phosphorus of the cereal diets became available, and their calcifying properties were definitely increased. The non-cereal diets, however, were still rachitogenic as the total phosphorus was low and, even if all absorbed, was not sufficient to prevent rickets. The difference in calcification exhibited by the animals on these diets therefore can be directly explained by a difference in the amount of utilizable phosphorus. In contrast to this, when phosphorus but no calcium was added to the diets, the extent of calcification was limited by the calcium. Inasmuch as all of the diets were nearly equal in their content of calcium, the degree of calcification would be expected to be approximately equal. Thus it is seen that when phosphorus is the limiting factor there is a difference in the calcification produced on the various diets because the amount of available phosphorus is not the same in each case. In contrast to this,

when calcium is the limiting factor and an excess of phosphorus is added, there is no appreciable difference in the calcification produced on the several diets because their calcium contents are all approximately the same.

SUMMARY AND CONCLUSIONS

Rolled oats, wheat, yellow corn, dextrinized cornstarch and sucrose have been compared with respect to their ability to promote calcification by feeding them as supplements to a basal mixture consisting of alcoholic extracted fibrin, carotene and a salt mixture complete except for calcium and phosphorus. With the calcium content of the diet high (3% calcium carbonate) and no phosphorus added, rolled oats and wheat definitely produced more calcification than did corn or either of the non-cereal rations. When the calcium carbonate was reduced to 1%, calcification on all of the cereal diets was considerably increased whereas there was no apparent increase associated with the dextrin or sucrose diets. Quite similar results were obtained when the calcium was omitted entirely, although the difference in calcification between the cereal and non-cereal diets was less.

When phosphorus was added to the low-calcium diets, there was no detectable difference in the calcification produced on any of the diets.

The above data are discussed in regard to the relation of the amount of calcium and available phosphorus in the diet to calcification in the rat.

No evidence was obtained in support of the view that cereal grains contain a specific anti-calcifying factor.

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THE DISTRIBUTION OF RIBOFLAVIN IN MEAT AND MEAT PRODUCTS [1, 2]

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The recognition of riboflavin as a specific member of the vitamin B complex necessitates a reappraisal of the available figures for its distribution in biological materials. Many of the earlier assays for the water soluble, heat stable vitamin were believed to be tests for only riboflavin, but they were, in reality, measures of more than one factor. Hoagland ('29) reported the antineuritic and water soluble B vitamin content of beef and pork. Hoagland and Snider ('30) estimated "the vitamin G in certain meats and meat products." The vitamin G in beef and veal has been determined by the rat growth method of Day ('31) and in the organs of lambs by Pierson ('34).

When crystalline riboflavin became available for standardization purposes, various spectrographic and colorimetric procedures were developed, thus providing additional figures for the occurrence of this compound in natural products. These methods have been criticized by Ellinger ('38). The crystalline riboflavin permitted the use of a definite standard in the biological methods which increased their accuracy. The values in the literature previous to 1934 on the distribution of riboflavin are not of a quantitative nature. In this paper we wish

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to report the amount of riboflavin in meats and meat products as determined by the microbiological method.

EXPERIMENTAL PART

These assays have been made by the bacteriological technic developed by Snell and Strong ('39). This method depends upon the growth of a specific strain of Lactobacillus casei on a media deficient in riboflavin. The basal media consists of 0.5% photolyzed, NaOH-treated peptone, 1% glucose, 0.01% cystine, a riboflavin-free yeast activator equivalent to 0.1% of the original yeast extract and a salt mixture. Five cubic centimeters of the basal media were pipetted into ordinary test tubes kept in a large metal rack. The supplement to be assayed and sufficient water to give a volume of 10 cc. were added to each tube.

The tissues assayed in this series were prepared for drying according to our previously described method and in many cases were the same samples as those used in our earlier work (Mickelsen, Waisman and Elvehjem, '39). The protein, fat and moisture analyses of samples 32 to 72 are given in the first paper, the values for the remaining samples are given in table 1. The riboflavin content of the material to be assayed was first roughly determined in a preliminary run so that a suitable weight of the sample could be used. As a result of this test, the kidney and liver were used at a level of 0.1 gm. and the other tissues at 1.0 gm. The accurately weighed tissue was placed in a test tube containing about 10 cc. of warm water. This was then homogenized in the apparatus described by Potter and Elvehjem ('36). The uniform mixture was then quantitatively transferred to a 50 cc. centrifuge tube and diluted to 35 cc. with water. These tubes were autoclaved at 15 pounds pressure for 15 minutes. As soon as the tubes cooled, they were centrifuged. The supernatant liquid was poured into a 100 cc. volumetric flask. The residue was suspended in about 20 cc. of water and centrifuged again. The washings were added to the original extract in the volumetric flasks. All extracts were finally diluted to

TABLE 1

Composition of some of the animal tissues used in these studies[1]

Meat	Meat process	Meat sample	Moisture in original sample	Fat in dried sample	Protein in dried sample
			%	%	%
Beef brain	Dried	77	...	45.3	44.6
Beef heart	Dried	73	78.7	8.4	73.6
Beef heart	Stewed	87	...	9.0	80.1
Beef kidney	Dried	81	77.2	11.5	73.0
Beef kidney	Dried	84	79.2	9.3	74.6
Beef kidney	Stewed	85	...	10.0	75.6
Beef kidney	Stewed	112	...	12.2	73.2
Beef liver	Dried	98	...	18.9	64.1
Beef liver	Dried	110	...	13.9	66.1
Beef liver	Fried	99	...	17.0	61.0
Beef liver	Fried	111	...	17.0	67.2
Beef lung	Dried	78	...	10.6	73.4
Beef pancreas	Dried	79	...	19.2	66.6
Beef pancreas	Dried	113	...	24.0	60.5
Beef round	Dried	105	72.3	12.8	71.6
Beef round	Fried	106	...	22.4	66.8
Beef round	Roasted	107	...	19.0	70.0
Beef spleen	Dried	76	...	9.0	66.3
Beef spleen	Dried	108	...	8.3	69.4
Beef spleen	Stewed	88	...	7.0	73.8
Beef spleen	Stewed	109	...	8.3	72.8
Beef tongue	Dried	82	72.2	42.4	49.4
Veal hindquarters	Dried	103	73.1	4.3	80.7
Veal liver	Dried	97	71.0	15.4	64.7
Lamb, leg of	Dried	80	74.2	14.0	74.3
Lamb liver	Dried	96	71.0	17.0	68.7
Pork ham	Dried	95	75.6	11.8	75.1
Pork ham	Dried	114	72.9	19.7	67.0
Pork ham	Dried	118	72.7	20.3	68.6
Boiled ham	Dried	101	67.1	12.2	61.6
Boiled ham	Dried	115	65.4	15.7	62.4
Smoked ham	Dried	102	71.5	11.6	59.3
Smoked ham	Dried	116	70.6	15.4	57.9
Tender ham	Dried	117	65.6	14.7	58.4
Tender ham	Dried	119	67.0	17.7	60.0
Pork heart	Dried	104	77.7	9.8	79.5
Pork kidney	Dried	83	78.1	13.2	73.0
Pork liver	Dried	86	67.8	9.7	54.1
Pork loin	Dried	74	71.8	24.4	66.2
Pork loin	Dried	89	66.6	33.4	59.5
Pork loin	Dried	90	71.4	9.7	81.6
Pork loin	Dried	91	70.0	24.4	63.6
Pork loin	Dried	92	70.1	34.5	56.0
Pork loin	Fried	93	...	32.6	59.6
Pork loin	Roasted	94	...	25.8	68.6

[1] For analyses of samples 32–73 see J. Nutrition, vol. 17, p. 272 ('39).

100 cc. and various aliquots of this solution were added to the basal media in the test tubes. In order to prevent the growth of microorganisms in the tissue extract, a small amount of chloroform was added to each sample. Control runs indicated that this and similar preservatives had no influence on the results of the method as long as care was exercised to prevent the addition of globules of chloroform to the test tubes. Usually, as a trial run, 0.5, 1.0 and 1.5 cc. of this solution were used. All assays were run in duplicate. In each series of assays standard tubes with graded levels of crystalline riboflavin were also set up. After the tubes were made up to volume, they were stoppered with cotton plugs and autoclaved for 15 minutes. Shortly after the tubes cooled, they were aseptically inoculated with a normal saline suspension of a freshly cultured Lactobacillus casei. The tubes were then incubated at 37° for 3 days. At the end of this period, the acidity in all tubes was determined by titration with N/10 NaOH. A reference curve was plotted from the titration values of the standard tubes containing the crystalline riboflavin. This curve was used in determining the riboflavin content of the unknown samples. Within a certain range, the amount of acid produced by the organism is directly proportional to the concentration of riboflavin in the media. All the work involved in the preparation of the extracts as well as the incubation was performed away from bright light so as to avoid a possible destruction of riboflavin in the aqueous solution.

Snell and Strong ('39) reported a series of assays on biological materials both by the bacteriological and rat growth methods. Both tests gave the same values for riboflavin which was conclusive evidence for the validity of the values secured by these assays. We have run similar parallel assays on some of our meats and meat products. The results secured by the rat growth and microbiological assays fully confirmed the previous results.

Simultaneous assays of the fresh and the dried material at equivalent levels showed that there was no destruction of

riboflavin during our drying process. A sample of pork liver (no. 122) was analyzed in both the fresh and the dried condition. The fresh sample was assayed by the bacteriological method shortly after the meat arrived from the abattoir. The same sample was also assayed as soon as it was dried. The fresh tissue contained 80 micrograms of riboflavin per gram on the dry weight basis, which agrees with the 85 micrograms per gram in the dry sample.

The results of the bacteriological assays (table 2) furnish additional confirmation for the high content of riboflavin in the livers and kidneys of all animals. Beef liver has varied between 105 and 125 micrograms of riboflavin per gram of dried material. Veal liver fell within the same range with a variation between 100 and 135 micrograms of riboflavin. Lamb liver, containing 90 micrograms and pork liver 80 to 90 micrograms per gram, showed a lower content of riboflavin. The dried beef kidney contained 90 to 100 micrograms per gram while pork kidney had 90 to 92 micrograms of riboflavin per gram. Next in potency came pork heart with 50 micrograms of riboflavin per gram while beef heart was slightly lower than this with 36 micrograms per gram. The pancreas of beef came next in order with 17 to 19 micrograms per gram followed by beef lung which contained 15 micrograms of riboflavin per gram. The remainder of the tissues including the muscular portions of the animals assayed contained approximately 10 micrograms of riboflavin per gram. One marked exception to this is the light meat of poultry, two samples of which contained only 3 micrograms of riboflavin per gram whereas one sample of dark meat contained 10 micrograms per gram.

In studying the influence of cooking on the riboflavin content of these tissues, a large cut of meat was secured and prepared according to our previously described method (Mickelsen, Waisman and Elvehjem, '39). One-half of it was cooked and then prepared for drying, whereas the other half was dried immediately. These studies revealed that stewing had no perceptible influence on the riboflavin content. The stewed beef kidney, stewed beef heart and stewed beef spleen

TABLE 2

The riboflavin content of meat and meat products

Meat	*Meat process*	*Meat sample*	*Flavin content / gm.* Fresh	Dry
Beef brain	Dried	57	2.5	12
Beef brain	Dried	77	...	11
Beef heart	Dried	73	7.6	36
Beef heart	Stewed	87	...	37
Beef heart	Stewed	54	...	34
Beef kidney	Dried	49	21.1	100
Beef kidney	Dried	81	22.8	100
Beef kidney	Dried	84	18.7	90
Beef kidney	Stewed	85	...	90
Beef kidney	Stewed	112	...	85
Beef liver	Dried	98	...	105
Beef liver	Dried	110	...	125
Beef liver	Fried	99	...	65
Beef liver	Fried	111	...	86
Beef lung	Dried	78	...	15
Beef pancreas	Dried	64	5.5	17
Beef pancreas	Dried	79	...	19
Beef pancreas	Dried	113	...	19
Beef round	Dried	40	...	9
Beef round	Dried	105	1.9	7
Beef round	Fried	42	...	6
Beef round	Fried	106	...	5
Beef round	Roasted	107	...	5
Beef spleen	Dried	59	3.5	15
Beef spleen	Dried	76	...	13
Beef spleen	Dried	108	...	16
Beef spleen	Stewed	88	...	13
Beef spleen	Stewed	109	...	15
Beef tongue	Dried	82	2.2	8
Veal hindquarter	Dried	56	2.4	10
Veal hindquarter	Dried	103	2.4	9
Veal hindquarter	Fried	45	...	11
Veal liver	Dried	70	...	100
Veal liver	Dried	97	39	135
Lamb, leg of	Dried	55	3.6	13
Lamb, leg of	Dried	80	2.8	11
Lamb liver	Dried	96	26	90
Pork ham	Dried	32	2.7	10
Pork ham	Dried	95	2.0	8
Pork ham	Dried	114	2.2	8
Pork ham	Dried	118	2.2	8
Boiled ham	Dried	101	2.6	8

TABLE 2 (*continued*)

Meat	*Meat process*	*Meat sample*	*Flavin content / gm.* Fresh	Dry
Boiled ham	Dried	115	2.8	8
Pork ham	Fried	52	...	9
Smoked ham	Dried	47	2.9	9
Smoked ham	Dried	102	2.3	8
Smoked ham	Dried	116	2.4	8
Smoked ham	Fried	48	2.6	8
Canned ham	Dried	72	2.2	7
Tender ham	Dried	117	2.4	7
Tender ham	Dried	119	2.0	6
Pork heart	Dried	104	11.2	50
Pork kidney	Dried	62	20	92
Pork kidney	Dried	83	19.7	90
Pork liver	Dried	86	29	90
Pork liver	Dried	122	26.9	85
Pork liver	Fresh	122	25.4	80
Pork loin	Dried	36	2	7
Pork loin	Dried	74	2.5	9
Pork loin	Dried	89	2.7	8
Pork loin	Dried	90	2.3	8
Pork loin	Dried	91	2.4	8
Pork loin	Dried	92	2.7	9
Pork loin	Baked	37	...	3
Pork loin	Fried	38	...	6
Pork loin	Fried	93	...	4
Pork loin	Roasted	94	...	3.5
Poultry, dark	Dried	66	2.6	10
Poultry, light	Dried	65	0.8	3
Poultry, light	Dried	68	0.7	3

had practically the same riboflavin content as the dried uncooked samples. When some of the samples were fried the riboflavin content varied considerably, with pork loin showing a destruction of approximately 50%. In the case of beef liver, the destruction was nearly 40% whereas about 33% of the vitamin was destroyed in fried beef round. The original uncooked samples were not available for purposes of comparison in the case of fried veal and pork loin but since a number of uncooked samples of these tissues contained nearly the same amount of riboflavin as the fried samples, it seems valid to conclude that there was very little destruction in both of these cases. Two cuts of meat were roasted. The beef

round after cooking showed a 30% destruction and the pork loin a 60% destruction of riboflavin. When pork loin was baked, about 60% of the original riboflavin was lost. It is difficult at present to explain the variation in the stability of riboflavin during different cooking processes. Further studies are being made in an effort to correlate the time of heating and the internal temperature of the meats with the stability of riboflavin.

DISCUSSION

From a comparison of the values for riboflavin in meats and meat products as reported in the literature and values secured by this method, it is evident that there is a close agreement in the results obtained with the rat growth and bacteriological methods. Darby and Day ('38) found 23.0 micrograms of riboflavin in fresh pork liver, while according to our procedure, there are 25.4 to 26.9 micrograms. These workers also found 2.8 micrograms in fresh lamb which agrees well with our values of 2.8 to 3.6 micrograms per gram. Similar agreement is apparent in the cases of cured and fresh hams. Charite and Khaustov ('35) by a colorimetric method found 35.6 micrograms of riboflavin in fresh calves liver whereas we found approximately 37 micrograms. Most of the values reported on the basis of the fluorometric method are considerably lower than ours. For instance, recent results reported by Schormüller ('39) are about one-half and in some cases only one-third of our values. The results reported by von Euler and Adler ('34 a, b) are also somewhat lower than those obtained in this study. The fluorometric method was used in both of these studies.

Sebrell and Butler ('38) have recently shown that certain patients, when restricted to the pellagra-producing diet of Goldberger, develop lesions around the mouth which are not cured by the administration of nicotinic acid but show a prompt response to pure riboflavin. This shows definitely that this vitamin is important in human nutrition. At present, no accurate figures are available on the human requirement. In 1937 Rose suggested that an adult should secure 20 Sherman-

Bourquin units of riboflavin per 100 calories. Since one Sherman-Bourquin unit is equal to 2.0 to 2.5 micrograms (Bessey, '38), the above value is approximately 1.0 to 1.2 mg. of riboflavin per day. Hogan ('38), in discussing Emmerie's work ('36) on the excretion of riboflavin in the urine of adults, concluded on the basis of the recorded data that the human adult required 2 to 3 mg. of riboflavin per day. The excretion studies were made on the assumption that all of the ingested riboflavin was absorbed and that none of it appeared in the feces or was utilized by intestinal bacteria, consequently these values may be slightly high.

On the basis of these values, we may assume that the human requirement for riboflavin lies between 1 and 2 mg. per day. The work reported in this paper conclusively shows that meats and meat products are an important source of this vitamin. The fried beef liver contained 65 and 86 micrograms of riboflavin per gram of dried material. In order to supply the suggested minimum daily requirement, approximately 12 to 15 gm. of dry liver or 50 to 60 gm. of the fresh material would be necessary. In the case of fried pork loin, approximately 450 gm. of fresh material would be needed to meet the daily demand for riboflavin.

SUMMARY

The riboflavin content of meats and meat products has been determined by the use of a microbiological method.

Liver and kidney of pork, beef, lamb and veal are uniformly higher than other organs of these species. The muscular tissues show a lower riboflavin content than the glandular tissues.

There appeared to be no loss of riboflavin by the ordinary household method of stewing. However, the samples that were roasted or fried showed appreciable losses.

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THE PRESERVATION OF THE 'GRASS JUICE FACTOR' IN SILAGE[1,2]

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FOUR FIGURES

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In 1934 it was shown by Elvehjem, Hart, Jackson and Weckel ('34), and Stirn, Elvehjem and Hart ('35) that milk produced on a regular winter ration was markedly inferior in nutritive value to milk produced by cows on summer pasture. Kohler, Elvehjem and Hart ('36, '37, '38) have shown that various grasses contain a factor (or factors) which is essential for maintenance and growth of rats ('36, '37) and guinea pigs ('38). Small supplements of such grasses enable guinea pigs on a mineralized winter milk diet to grow normally. Thus the seasonal changes in the nutritive value of milk can be related to the presence of a factor in the forage ingested by the cows; that is, during the summer the animals on pasture obtain sufficient 'grass juice factor' not only for body needs but also for transmission into the milk. It has been possible to decrease the growth-promoting value of milk at any time of the year by placing cows on a dry ration (winter ration) for several months.

It follows that by feeding forages rich in the 'grass juice factor' cows would produce a winter milk comparable to

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summer milk in nutritive value. The problem then arises of preserving forages rich in the 'grass juice factor' for winter feeding. Since silage appears to be both a logical and practical means of carrying the factor, we have studied the preservation of the 'grass juice factor' in various types of silages. In this paper we wish to report the growth of guinea pigs on mineralized winter milk supplemented with various silages; and also on the mineralized winter milks from groups of silage-fed cows.

EXPERIMENTAL PROGRAM

Guinea pigs weighing approximately 300 gm. each were used. They were fed a basal diet of mineralized milk. The milk used was obtained fresh each morning from the same cow (Holstein) at the dairy barn of the university. This cow had been fed a winter ration low in the 'grass juice factor' for several months. Various kinds of silage plus a mineral supplement consisting of 1 mg. Fe as ferric pyrophosphate, 0.1 mg. Cu as copper sulphate, and 0.1 mg. Mn as manganese sulphate were fed. The silage supplement together with the mineral solutions was fed mixed with approximately 2 cc. of orange juice each morning. The orange juice was used to increase the palatability of the silage. Kohler, Elvehjem and Hart ('36) have shown that 2 cc. of orange juice daily, when added to mineralized winter milk, has no growth-promoting effect on guinea pigs.

In the afternoon, when the animals had completely consumed the supplements, enough milk was fed to allow ad libitum feeding. The animals were weighed each day and their weights recorded. Control guinea pigs were fed a diet consisting of the whole milk supplemented with the minerals.

Four kinds of forage were ensiled as follows:

(1) Alfalfa (June cutting) ensiled with 60 pounds molasses per ton.
(2) Alfalfa (June cutting) ensiled with 15 pounds phosphoric acid (79%) per ton.
(3) Clover and timothy in about equal parts (June cutting) ensiled with 60 pounds molasses per ton.
(4) Soy beans (October cutting) ensiled with 60 pounds molasses per ton.

Silages were also put up in milk bottles in the fall with clover, alfalfa, Kentucky blue grass and dried oat grass (dehydrated oat grass no. 1200-2 was supplied by the Cerophyl Laboratories). A. I. V., molasses and phosphoric acid silages were prepared from the fresh materials and A. I. V. silage from the dehydrated oat grass. A calculated amount of preservative in 50 cc. of water was added to 1 kg. of fresh plant material, and thoroughly mixed by hand. In the case of the dried grass, it was first mixed with water to form a paste. The amounts of preservative used per kilogram were:

(1) A. I. V. silage: 37.5 cc. of 2N acid (1 mol. H_2SO_4 + 1 mol. HCl); equal to 34 liters of acid per ton.

(2) Molasses silage: 32 gm. molasses; equal to 64 pounds per ton.

(3) Phosphoric acid silage: 15 gm. 79% H_3PO_4; equal to 30 pounds acid per ton.

The mixtures were packed tightly into milk bottles, closed with rubber stoppers containing Bunsen valves, and set aside. Each bottle contained approximately 750 gm.

At the time of cutting, samples of the soy bean, clover, alfalfa (third cutting) and Kentucky blue grass were prepared for feeding by drying 16 hours at 40°C. When dry they were ground and stored in the refrigerator. It has been shown by Kohler, Elvehjem and Hart ('38) that the activity of dried grasses disappears on storage at room temperature but that it is relatively stable at lower temperatures.

After the bottle silages had been allowed to stand for approximately 2 months, they were opened and the contents dried, ground and stored in the same manner as the fresh materials. Guinea pigs were fed these materials at a level of 3 gm. per day as a supplement to the basal mineralized winter milk. Because of the low 'grass juice factor' content of the dried fresh alfalfa (fig. 1) it seemed unnecessary to test the alfalfa bottle silages.

In figures 1 and 2 are given the growth curves of the guinea pigs fed the basal mineralized winter milk alone and plus the supplements of the materials which were fed both as dried fresh forages and as dried silages.

After about 6 months the University farm silages were sampled and tested in the same way as the bottle silages. The growth curves for these animals are given in figure 3.

The 'grass juice factor' content of the silage milks. The growth-promoting properties of milks from cows receiving the phosphoric acid alfalfa, the molasses alfalfa and the molasses clover-timothy silages were studied. Early in November,

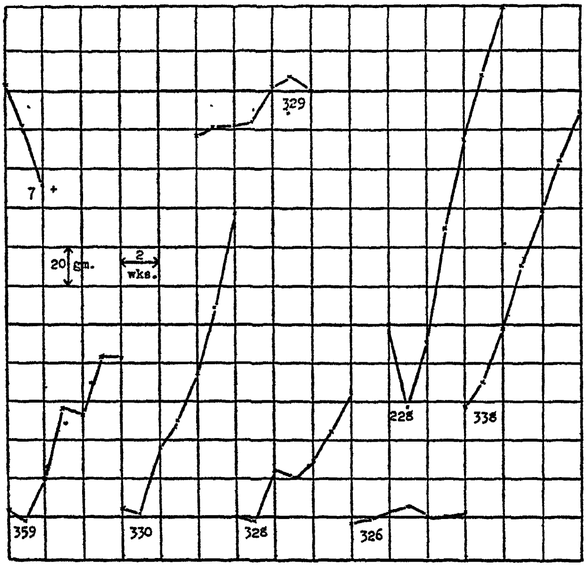

Fig. 1 Growth curves of guinea pigs fed mineralized winter milk plus supplements (3 gm. per day) of dried fresh forages and silages as follows: No. 7 control, no. 329 fall alfalfa, no. 228 dehydrated oat grass (fed at a 2-gm. level), no. 338 A.I.V. oat grass bottle silage, no. 359 Kentucky blue grass, no. 330 A.I.V. Kentucky blue grass bottle silage, no. 328 phosphoric acid Kentucky blue grass bottle silage, no. 326 molasses Kentucky blue grass bottle silage.

1938, three groups of cows were placed on experiment as follows: Groups 1 and 2 consisted of 1 Holstein, 2 Guernseys, 1 Jersey and 1 Brown Swiss; and in group 3 there were 2 Holsteins, 2 Guernseys and 1 Brown Swiss. The ration was made up of 42 pounds silage, 6 pounds alfalfa hay and 10.5 pounds grain mixture per day. Group 1 received the molasses

clover-timothy silage, group 2 the molasses alfalfa silage, and group 3 the phosphoric acid alfalfa silage.

Early in January, 1939 (i.e., 2 months after the cows had been placed on the silage ration), the feeding of the milks to the guinea pigs was started. All the milk from one milking of the cows in one group was mixed together and a quart aliquot taken. This was done for each group every day. Two

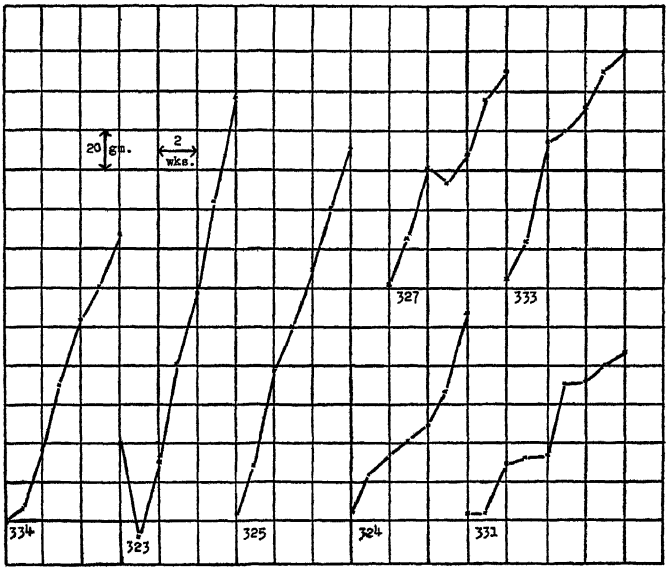

Fig. 2 Growth curves of guinea pigs fed mineralized winter milk plus supplements (3 gm. per day) of dried fresh forages and silages as follows: No. 327 soy bean, no. 333 molasses soy bean University farm silage, no. 334 red clover, no. 323 A.I.V. clover bottle silage, no. 325 phosphoric acid clover bottle silage, nos. 324 and 331 molasses clover bottle silage.

animals were fed each milk. They were fed a small amount of the milk plus the added minerals in the morning and after this had been consumed they were given an excess of milk. The growth curves of these guinea pigs are given in figure 4. Included for comparative purposes is the growth of a guinea pig on a good summer pasture milk, taken from the work of Kohler, Randle and Wagner ('39).

In order to determine if orange juice had any effect as used with the silage, one guinea pig was placed on milk from group 1 plus 10 cc. of orange juice daily. The curve of this animal (no. 17) is also plotted on figure 4. The pig died during the fifth week and showed gastric ulcers.

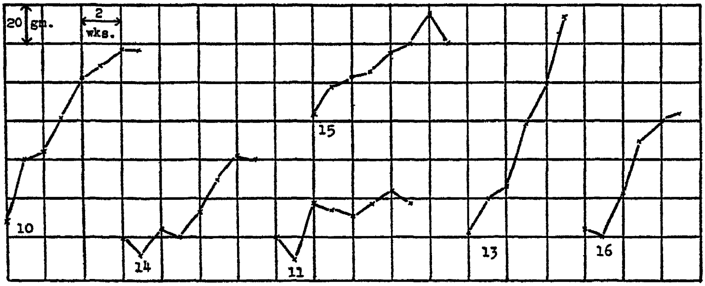

Fig. 3 Growth curves of guinea pigs fed mineralized milk plus supplements (3 gm. per day) of dried silage from the University farm as follows: Nos. 10 and 14 molasses clover-timothy silage, nos. 11 and 15 molasses alfalfa silage, nos. 13 and 16 phosphoric acid alfalfa silage.

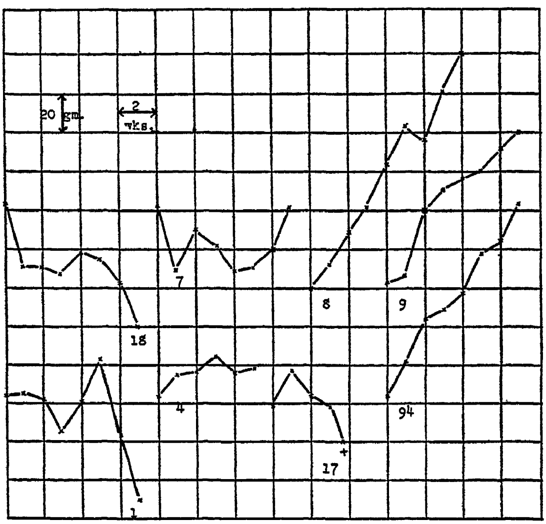

Fig. 4 Growth curves of guinea pigs receiving various mineralized milks as follows: No. 94 summer pasture milk, nos. 1 and 18 molasses clover-timothy silage milk, nos. 4 and 7 molasses alfalfa silage milk, nos. 8 and 9 phosphoric acid alfalfa silage milk, no. 17 molasses clover-timothy silage milk plus 10 cc. orange juice per day.

Animals 1, 2 and 4 (figure 4) after 6 weeks began to show loss of hair typical of a flavin deficiency and thereafter all the other guinea pigs on these milk diets were given a supplement of 50 micrograms of flavin per day. With this added flavin there were no cases of loss of hair and the weight seemed to be better maintained.

DISCUSSION

The results presented indicate that the 'grass juice factor' is definitely retained in plants after ensiling. This fact is obvious from a comparison of the growth curves of the animals on silage supplements with the control animal. However, it is obvious that there is a marked variation in the amount retained and that the degree of preservation is dependent upon the ensiling procedure used. It is evident that the acid-prepared silages were superior to the molasses-prepared silages in 'grass juice factor' content. Among the acid-prepared silages, the A. I. V. seems to be superior.

The reason for the lower potency of the molasses silages as compared to the acid-prepared silages may be due to destruction of the 'grass juice factor' during the initial fermentation which takes place in this type of silage.

Under farm conditions, fair retention is found even in the molasses-preserved silages, as is shown by comparison of the dried fresh and ensiled soy beans (fig. 2). Also the clover-timothy and alfalfa molasses silages gave some growth (fig. 3).

The potency of plant materials seems to vary with the stage of growth. The mature plants have been found much less effective than rapidly growing ones. The effect of maturity is shown by the good growth obtained on the young alfalfa cut in June (fig. 3) as compared to the poor growth obtained on the fall alfalfa cut in October (fig. 1) which was more mature. Ordinarily farmers allow their hay crops to reach a mature stage before harvesting, and at this stage the 'grass juice factor' content is low. This fact together with the destruction of the active principle during drying and storage accounts for the low potency of winter milk. However,

by acid ensiling at an immature stage, this factor is well preserved.

Comparison of various materials ensiled seems to indicate a very high 'grass juice factor' content for clover, young oat grass and for soy beans, a fairly high content for Kentucky blue grass and a lower content for alfalfa. It is difficult, however, to draw definite conclusions due to differences in state of maturity and time of harvesting of the various materials.

Figure 4 illustrates the fact that a winter milk high in the 'grass juice factor' was obtained by feeding the phosphoric acid alfalfa silage to the cows. By comparing guinea pigs no. 8 and no. 9 with no. 94 (fig. 4), it is seen that this phosphoric acid silage-produced winter milk is approximately equal to good pasture-produced summer milk. Comparing the growth curves on figure 4 with the control animal (no. 7, fig. 1), it seems that all of the silage-produced milks contain more of the factor than a dry ration produced winter milk. In this connection, it is interesting that Riddell et al. ('36) in studies on the vitamin C content of milk showed that 40 cc. of pasture milk, fed as a supplement to the basal scorbutic ration, produced much better growth in guinea pigs than did a similar supplement of milk produced by cows on a dry ration, and cows receiving silage produced a milk of intermediate growth-promoting qualities. Also Peterson, Bird and Beeson ('37) and Hegsted et al. ('39) found that rats fed mineralized milk produced by cows fed A. I. V. alfalfa silage grew more rapidly than rats fed mineralized winter milk and that there was no appreciable difference in the growth of rats fed milk produced from A. I. V. alfalfa silage, green alfalfa or pasture.

It is probable that the early observation that summer pasture milk was richer in vitamin C than winter milk was really a difference in the 'grass juice factor.'

SUMMARY

1. It was found possible to preserve the 'grass juice factor' in forages by ensiling.

2. The A. I. V. and phosphoric acid methods of ensiling were found to give better preservation of the 'grass juice factor' than did the molasses method.

3. Milk from cows fed phosphoric acid alfalfa silage was found to be approximately as rich in the 'grass juice factor' as milk produced by cows on summer pasture.

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THE USE OF ISOLATED RADIATION IN EXPERIMENTS WITH THE RAT

II. EFFECTS OF DARKNESS, VISIBLE, AND INFRA RED RADIATION ON THREE SUCCEEDING GENERATIONS OF RATS

(*a*) GROWTH AND STORAGE OF VITAMIN A[1]

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THREE FIGURES

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INTRODUCTION

Jones ('29) has described a series of filters for the isolation of spectral bands in the ultra-violet, visible and infra red regions of the spectrum. A report of experiments made with the use of these filters has also been published (Luce-Clausen, '29). As an outcome of this work a series of four rooms was designed to provide a radiation environment in which rats could be studied, in suitable numbers, over long periods of time. A description of these rooms, with data on wave length limits and energy values of radiation, has been given by Jones and Tuttle ('39). The rooms will be designated:

Room I—Complete darkness
Room II—Near infra red radiation
Room III—Visible radiation
Room IV—Far infra red radiation

Experiments were started in September, 1936, and were concluded in the spring of 1938. Studies were made of growth, storage of vitamin A, and of reproduction. This paper reports results on growth and storage of vitamin A.

[1] Aided by a grant from the Committee on the Effects of Radiation upon Living Organisms, Divisions of Biology and Agriculture, National Research Council.

EXPERIMENTAL PROCEDURE

At the beginning of the experiment sixteen pregnant females of the Experimental Colony strain, were shipped to us from The Wistar Institute. These rats were, within the limit of 4 days, of the same age; one group of five and another of six were litter mates. We distributed them, four in a room, sampling them so that there were, as far as possible, litter-mate rats from the sixteen, in each room. The mothers, after the young were weaned, were discarded. The young were raised in the different rooms and bred through three generations.

The rats were housed on tables placed immediately under the duct which carried the radiation equipment in each room. We tried, as far as possible, to eliminate all radiation from the dark room. It contained no radiator. The outlet duct from the radiation rooms (see diagram Jones and Tuttle) was in the dark room, but was well insulated, never more than slightly warm to the touch, and situated in a part of the room remote from the rat cages. The rats were radiated for 12 hours each day, 5 A.M. to 5 P.M., the lamps being turned on and off automatically by the use of a Sangamo time switch.

Rats were weaned on the twenty-eighth day. The original mothers were fed the Sherman B diet (Sherman and Campbell, '24) plus 10% meat scrap until the young were weaned. The young were then divided, in each room, into two groups. One group was reared on the Sherman B, the other on the 'McCollum' diet (Evans and Bishop, '22). We used the Sherman B diet because it was the one used in other experiments. The McCollum diet was chosen as being the best and most standard diet for studies on reproduction. The animals were fed ad libitum.

Unfortunately the commercial casein used in the 'McCollum' diet was found, after a period of a year, to be contaminated with fluorine. We have published (Hodge, Luce-Clausen and Brown, '39) an account of the teeth of the rats in this group and indicated, as a result of this accident, our findings as to the effect of darkness on fluorosis.

In analyzing results we have taken the presence of fluorosis into account, and have given results on each diet separately. The 'McCollum' diet has been indicated in quotation marks to signify the diet plus its contamination.

As a routine procedure all mother rats were fed a total of 1300 units of vitamin A in Haliver oil during lactation.

Records of daily growth during lactation, and of weekly growth after weaning were kept.

RESULTS

Growth (fig. 1)

The points on the curves represent mean weights for varying numbers of rats as shown in table 1. The numbers given are those at the beginning and at the end of the experiments. They diminish because some of the rats were killed on the seventeenth and fifty-fourth days to permit determination of the amounts of stored vitamin A; also only males needed for breeding were kept. Although the number used for the adult curves is not large, the results are very consistent.

The upper curves (fig. 1) represent early growth from birth to weaning. The lower set show the growth records of male rats up to adult life. In all cases the results in the two infra red rooms were so nearly alike that they have been combined in one curve. Lack of space prevents us from publishing all our growth curves. Those included demonstrate certain tendencies with which we have been impressed.

1. An effect of radiation is indicated because the same order is maintained throughout, namely, the curves for rats deprived of light show the slowest rate of growth (I), those for rats receiving visual radiation a faster rate (III), while the results for rats exposed to infra red radiation, in every case, are intermediate.

2. Sherman B rats. The difference in rate of growth between the various groups is first indicated in the second generation, beginning at about the fifth day and being maintained throughout the 15 weeks of observation. In the third genera-

tion this divergence is increased, partly due to a progressive decline of the growth rate of the rats in room I and partly due to a progressive improvement of growth of those in room III.

3. For rats on the 'McCollum' diet we see similar but intensified divergences. These are due mainly to failure of growth of the rats in room I, and maintenance of growth of those in room III. The early growth of the rats in room I is strikingly lower than that of the second and third generation rats fed

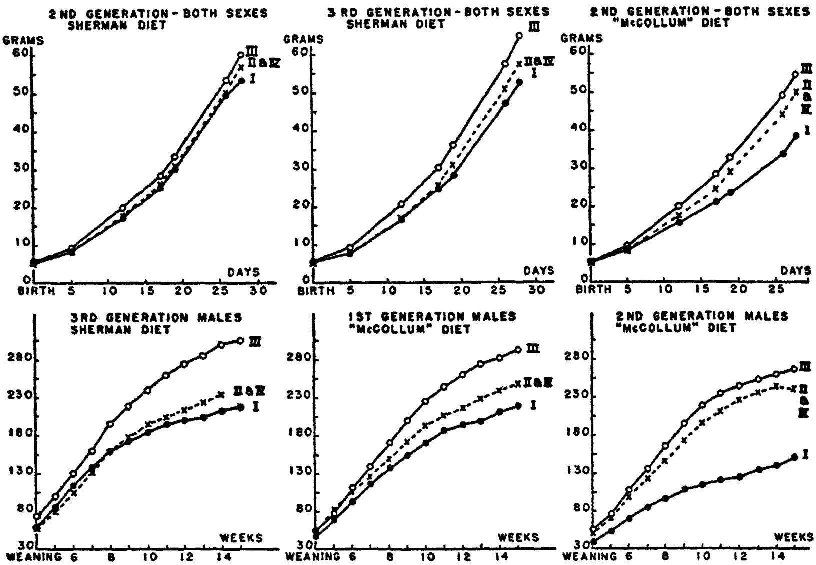

Fig. 1 Growth. I—rats in complete darkness. II and IV—rats exposed to infra red radiation. III—rats exposed to visible radiation.

the Sherman B diet. We attribute this to the effect of fluorine in the diet. Fluorine has not affected the early growth of the radiated animals to any great extent.

The later growth of the first generation male rats is strikingly similar to that of the third generation male rats on the Sherman B diet. In the second generation the radiated rats on this diet grew nearly as well as they did in the first generation, although there was a marked failure of growth in the rats in room I. Growth was maintained by the radiated animals even in the third generation.

4. Our results on female rats, which are not included, indicate a definite but less potent effect of radiation on the growth of female than on the growth of male animals.

TABLE 1

Number of rats used for growth curves[1]

ROOMS	BIRTH TO WEANING SHERMAN DIET 2ND GENERATION	BIRTH TO WEANING SHERMAN DIET 3RD GENERATION	BIRTH TO WEANING 'MCCOLLUM' DIET 2ND GENERATION
I	34–18	24–15	70–31
II and IV	67–36	68–27	152–58
III	42–25	27–14	40–24
	ADULT MALES SHERMAN DIET 3RD GENERATION	ADULT MALES 'MCCOLLUM' DIET 1ST GENERATION	ADULT MALES 'MCCOLLUM' DIET 2ND GENERATION
I	5–3	9–9	16–4
II and IV	9–4	15–9	25–7
III	5–2	8–4	8–5

[1] The numbers given are those at the beginning and at the end of the experiments (see text).

Vitamin A storage

Representative rats from all the rooms, on both diets, were killed on the seventeenth and fifty-fourth days and their livers analyzed for vitamin A. Similar analyses were made of all adult rats at autopsy. The method used for the vitamin A determinations was the modification of the Price-Carr method of S. W. Clausen (McCoord and Luce-Clausen, '34).

Figure 2 gives the units [2] of vitamin A in the liver plotted against the weight of the rat, with respect to both sexes at 17 days, males at 54 days, and adult males in all the groups. Figure 3 gives results for females.

In figures 2 and 3 the position of the line for each group of rats was determined by the method of least squares.

It is evident that in plotting the vitamin A values at given ages, the weight of the rat should be taken into account since these lines show a general upward slope with increased weight, at any given age. In cases, however, where we fed supple-

[2] One of our units = 3.8 international units.

ments of vitamin A to mother rats during lactation, this relationship of storage to body weight did not hold. The vitamin A values given in figures 2 and 3 were obtained from rats to whom no supplement had been fed.

The rats used in these determinations were taken from all three generations, but predominantly from the second and third.

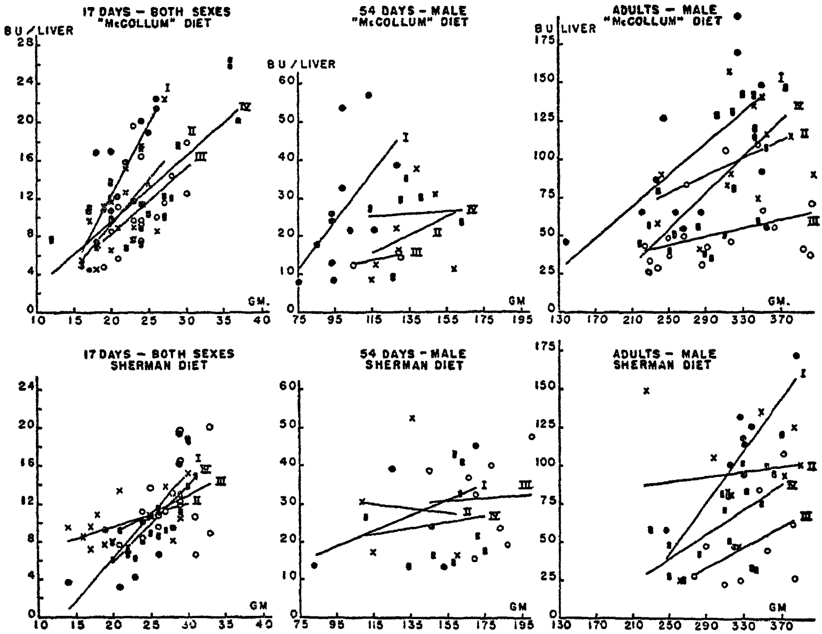

Fig. 2 Vitamin A in livers plotted against body weight. I—rats in complete darkness. II and IV—rats exposed to infra red radiation. III—rats exposed to visible radiation.

In considering the trend of these lines we were struck by the fact (fig. 2) that in six separate groups of observations the rate of increase of vitamin A with weight is greater with the animals in room I. The line for adult males in room IV ('McCollum' diet) runs parallel with that of room I, but the amount of vitamin storage, for any given weight, is greater in room I.

In figure 3 (females) the lines for rooms II and IV are similar to those of room I, but all are widely different from that of room III.

We decided therefore to compare the extremes, rooms I and III, which represent the wide difference between an environment of total darkness and one of visible radiation. Although at first sight, in view of the wide variations that are normally obtained in determinations of vitamin A, the difference in

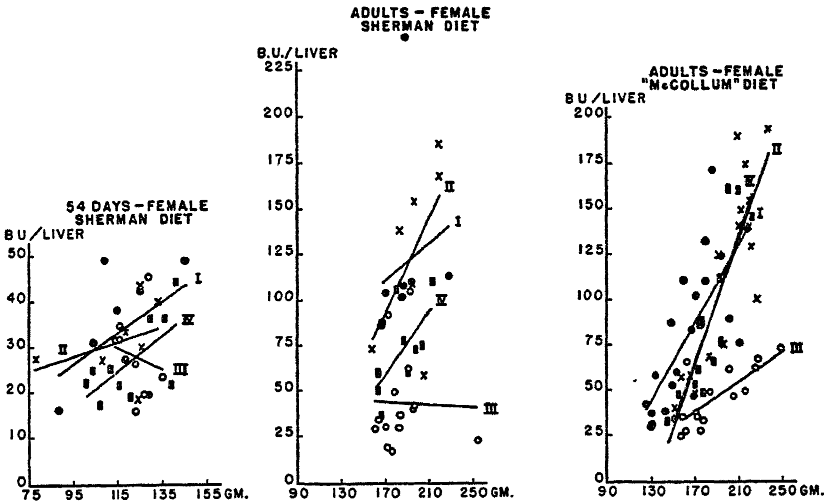

Fig. 3 Vitamin A in livers plotted against body weight. I—rats in complete darkness. II and IV—rats exposed to infra red radiation. III—rats exposed to visible radiation.

values for room I as compared with those for room III might not seem significant, the regularity with which they appeared in nine different groups of observations made further statistical analysis seem desirable. The method of analysis was as follows. The slope of each line was designated (b). The standard error (ε_b) was calculated for each slope.[3] The standard error of the differences of the slopes [4] for rooms I and III ($\varepsilon_{b_I - b_{III}}$)

[3] $\epsilon_b = \sqrt{\frac{1}{(N-2)}\left[\frac{N\,\Sigma\,y^2 - \Sigma^2\,y}{N\,\Sigma\,x^2 - \Sigma^2\,x} - b^2\right]}$

y = ordinate; x = abscissa; b = slope.

[4] $\epsilon^2_{b_I - b_{III}} = \frac{(N_I - 2)\,\epsilon^2_I}{(N_{III} - 2)} + \frac{(N_{III} - 2)\,\epsilon^2_{III}}{(N_I - 2)}$

was then calculated. The ratio of the difference between slope I and slope III over the standard error of the difference was then found. The probability of obtaining a ratio as large as, or larger than this, by pure chance was then determined by 'Student's' t table.[5]

Results as analyzed from figures 2 and 3 are given in table 2. It is seen that in one case (Adult females, 'McCollum' diet) the difference in slopes is clearly significant (0.008); in two cases (17 days and adult males, 'McCollum' diet) of borderline significance (0.025, 0.025); the remainder are not individually significant. Multiplying together the probabilities given in the table, for each sex and diet, we find that the chance is

TABLE 2

Probabilities that observed differences in slope are not significant

DIET	BOTH SEXES	MALES		FEMALES	
	17 days	54 days	Adult	54 days	Adult
Sherman B	0.10	0.35	0.10	0.15	0.25
'McCollum'	0.025	0.30	0.025	...	0.008

well under 1 in 100 that the slope difference is not significant throughout the life cycle (Sherman B, male and female, 4 chances in 1000; 'McCollum,' male and female, 2 chances in 10,000). Therefore in each case there is a significant slope difference at some one or more stages of the life cycle. It would seem therefore statistically justified to consider the different values for room I as compared with room III as not being chance variations within both groups, but as true differences in rates of increase of vitamin A, at given ages, in the absence or presence of radiation in the rats selected.

In this analysis we note, what we have found in many of our analyses, that the presence of fluorine in the diet intensifies an effect produced in rats fed the uncontaminated diet.

[5] "Statistical Methods for Research Workers" by R. A. Fisher, 7th edition, published by Oliver and Boyd, Edinburgh, 1938, p. 177.

DISCUSSION

Growth

Sherman B diet. The literature on the effects of radiation upon the growth of organisms is conflicting. Rats have been shown to grow equally well in darkness as in well-lighted rooms when the diet is optimal (Goldblatt and Soames, '23). On the other hand, Ludwig and von Reis ('31) report increased growth of rats reared under visible red radiation. Brown ('28) studied light environment and its effect on rabbits and demonstrated a growth-promoting effect of exposure to neon light which was greater than in animals receiving sunlight through window glass, or those reared in darkness.

One hesitates, in the absence of more quantitative feeding trials, to lay too much stress on growth curves. There seems, however, little doubt that in our experiments an effect of visible radiation, and, to a lesser degree, of infra red radiation in promoting growth has been demonstrated.

This effect is most marked in the third generation. The effect appears during lactation: the birth weight in all groups is identical but the curves for 'light' and 'dark' rats diverge after the fifth day. The intermediate position between 'dark' and 'light' of the two groups kept in infra red radiation makes it difficult to rule out an effect of these radiations. These results could be interpreted to mean some failure of function in the anterior hypophysis in animals deprived of light, resulting in lessened production of the lactogenic hormone in the mother, or of growth hormone in the young, an effect which according to some authors may be due to lack of retinal stimulation by light (Bissonnette, '38). Our results, although suggestive, afford no proof of this.

'McCollum' diet. We have no means of knowing the amount of fluorine ingested by the rats on this diet. It was sufficient to produce a chronic fluorosis. There is abundant evidence in the literature that fluorine added to the normal diet of rats inhibits growth (Iowa Agricultural Experiment Station reports, '28, '29; Goldemburg, '27; McClure and Mitchell, '31).

The growth inhibitory effect of fluorine in rats deprived of light is clearly seen in our curves. It is interesting to note, however, that radiation, both visual and infra red, overcomes, to a marked extent, the growth-inhibiting effects of fluorine. We have already described a lack of pigment in the teeth, due to fluorine, which occurred earlier in the group of rats deprived of light (Hodge, Luce-Clausen and Brown, '39).

Vitamin A storage—both diets

If we admit the validity of the statistical methods applied to our data, and it seems justifiable to do so, we are surprised to find that the rate of vitamin A storage, with increased weight at given ages, is faster in the groups of rats deprived of light than in those exposed to visible radiation, though the trend of the slope for increased weight is upward in both groups. The presence of fluorine in the 'McCollum' diet seems only to magnify the effect observed in the groups fed the uncontaminated diet.

In considering the factors which might decrease the storage of vitamin A in the 'light room' as compared with the 'dark room' rats several conditions may have been operative.

(1) Some increased use of vitamin A for bodily functions such as restoration of tissues, growth, and cell metabolism in the 'light room' rats, the males especially.

(2) A faster disappearance of vitamin A from the skin of the irradiated rats.

(3) Heat. McCoord ('38) has shown that hyperthermia and fever cause the supply of vitamin A to disappear from the liver. The rats in room III, especially during the summer months, were, from time to time, exposed to higher temperatures than were those in room I. This may have produced an effect on activity and appetite but the amount of heat was not sufficient to raise rectal temperature.

(4) Retinal stimulation. Tansley ('31) studied the mechanism of dark adaptation in albino rats. Using an illumination of 400 foot candles she found that ½ hour's exposure was sufficient to cause complete bleaching of the retina.

The only illumination used in our dark room was that from a single 7.5 watt green Mazda lamp placed 5 feet away from the cages. This was turned on for a short time each day for routine procedures. No rat was ever exposed to it for a longer time than that required to clean a cage, to weigh, or, in the case of females, to take a vaginal smear.

Measurements taken with a Weston foot candle meter, model 614, showed zero f.c. at 10 inches and a maximum of 16 f.c. at a distance of 0.5 inches. It therefore seems safe to say that the retinas of our animals were never, at any time, bleached by light. In room III the illumination was 300 f.c. and rats were exposed to it for 12 hours daily.

The researches of Wald ('35) have shown that small amounts of vitamin A are used up in the retina, and that light adapted retinas contain about 0.2–0.3 micrograms of vitamin A; dark adapted ones, only a trace. The ability of the retina to form visual purple after exposure to light depends on the supply of vitamin A. The turnover of vitamin A in the retina depends on the exposure of the retina to light. It is reasonable to suppose that complete absence of retinal stimulation, in a group of rats deprived of light, might lead to some retention of vitamin A that, under normal conditions of retinal stimulation by light, would be used up in the retina.

No quantitative data as to the amount of vitamin A used in retinal stimulation by light are available. Neither do we know whether the constant circulation of vitamin A in the retina would utilize enough to deplete liver stores, or, in the absence of stimulation, to augment them. It may be suggestive that the difference of storage between animals in rooms I and III is small, and for this reason more readily explainable on this basis.

CONCLUSIONS

1. Radiation in the visible region of the spectrum promotes growth in rats fed the Sherman B diet, as compared with groups fed the same diet and kept in complete darkness. This effect is more evident in the third generation of rats bred under controlled conditions of light, and more marked in males than in females.

2. Radiations in the near and far infra red regions have a growth-promoting effect which is definite but lesser in degree than that of visible light.

3. Visible radiation, and, to a lesser degree, infra red radiation, compensate for the marked inhibition of growth due to fluorosis seen in rats deprived of light.

4. Rats kept in darkness and fed the Sherman B diet, and also groups fed a diet contaminated with fluorine are shown to store vitamin A in livers at a more rapid rate for weight at given ages than do comparable groups exposed for 12 hours a day to visible light. It is suggested that lack of retinal stimulation by light may play a part in this result.

In conclusion, our thanks are due to the following: The Eastman Kodak Company for continued interest, support, and collaboration; the General Electric Company and the Westinghouse Company for special cooperation in regard to the supply of lamps; and the Corning Glass Company for interest and kind cooperation.

We also wish to thank Dr. Donald R. Charles for suggesting the mathematical methods employed in the analysis of data and for much helpful criticism; and Dr. Nancy Morey and Miss Eugenia Sheridan for help in the routine care of animals.

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THE USE OF ISOLATED RADIATION IN EXPERIMENTS WITH THE RAT

III. EFFECTS OF DARKNESS, VISIBLE AND INFRA RED RADIATION ON THREE SUCCEEDING GENERATIONS OF RATS

(*b*) REPRODUCTION [1]

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SIX FIGURES

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INTRODUCTION

In an earlier paper ('39) we have described results on growth and storage of vitamin A on three succeeding generations of rats kept continuously in the following environments: (a) darkness, (b) visible radiation, (c) infra red radiation. A full description of the rooms used in these experiments has also been given (Jones and Tuttle, '39).

This paper deals with the results on reproduction in the same rats. The rooms are designated room I (dark), room II (near infra red), room III (visible), and room IV (far infra red).

EXPERIMENTAL PROCEDURES

The date of opening of the vagina was carefully noted in all female rats. Daily records of vaginal smears, taken according to the technique of Long and Evans ('22), were kept, to determine the number of oestrous cycles up to the date of mating. Eight females, four on each diet used, the Sherman B (Sherman and Campbell, '24) and McCollum (Evans and Bishop, '22), were selected as breeders, along with four males, two on

[1] Aided by a grant from the Committee on the Effects of Radiation upon Living Organisms, Divisions of Biology and Agriculture, National Research Council.

each diet, in each room. Oestrous records were kept on all remaining female rats. For smearing we used for each rat an individual glass spatula sterilized daily.

At the age of 110 days, each female in the pro-oestrous stage, was placed with a male. The presence of vaginal plug and of sperm were taken as evidence of positive mating. Smears were taken till the fourteenth day when the appearance of a clot gave evidence of implantation. Smearing was then discontinued until after the birth of young; the animals were weighed daily during the last week of pregnancy. The number of young born were counted, and each litter was weighed daily for the first 17 days.

Results on the two diets are given separately. As explained in the previous paper an accidental contamination with fluorine of the commercial casein used in the McCollum diet was discovered and described (Hodge, Luce-Clausen and Brown, '39). The designation 'McCollum' signifies the diet plus its contamination.

RESULTS

1. Age of opening of the vagina, oestrous cycles

The age of opening of the vagina in all female rats is given in frequency polygons, those for the rats fed the Sherman B diet being shown in figure 1, and those for the 'McCollum' diet in figure 2. Details of oestrous cycles of females fed the same two diets are given in figures 3, 4, 5.

The total number of observations in the three groups given in figure 1 is 105, in figure 2, 135. The results for rats in rooms II and IV were so nearly identical that, for the sake of clearness, they have been plotted together.

Table 1 gives a summary of data obtained on the age of opening of the vagina in the six groups. It shows that on both diets the age of opening of the vagina is even earlier than normal [2] in rats receiving visible radiation (room III), the

[2] Since these experiments were concluded we have studied the age of opening of the vagina in ninety-three rats, of the Sprague-Dawley strain, fed the Sherman B diet and kept under ordinary conditions of light in our laboratory. The average age for these rats is 45.9 ± 0.4 days—or about the same as was obtained in rooms II and IV. This indicates that the 42-day figure for rats in room III is earlier than might be expected for rats kept under our experimental conditions.

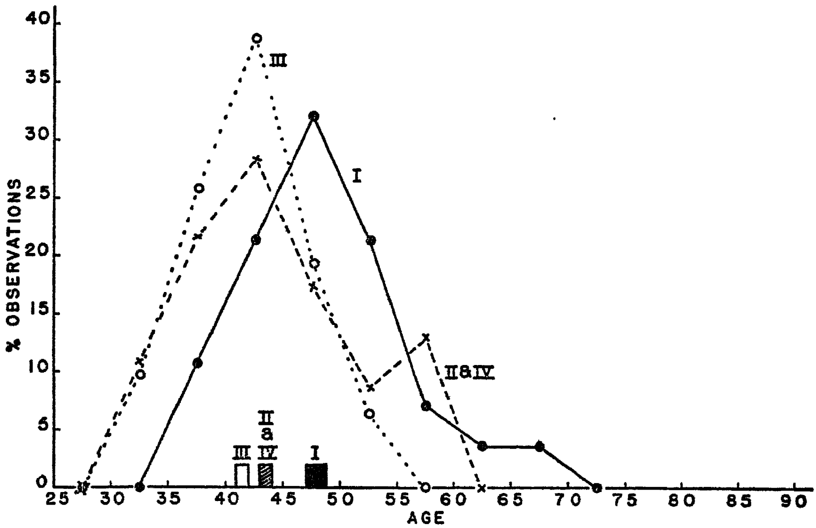

Fig. 1 Frequency polygons of age of opening of vagina. Sherman B diet. I = rats kept in complete darkness; II and IV = rats exposed to near and far infra red radiation; III = rats exposed to visible radiation.

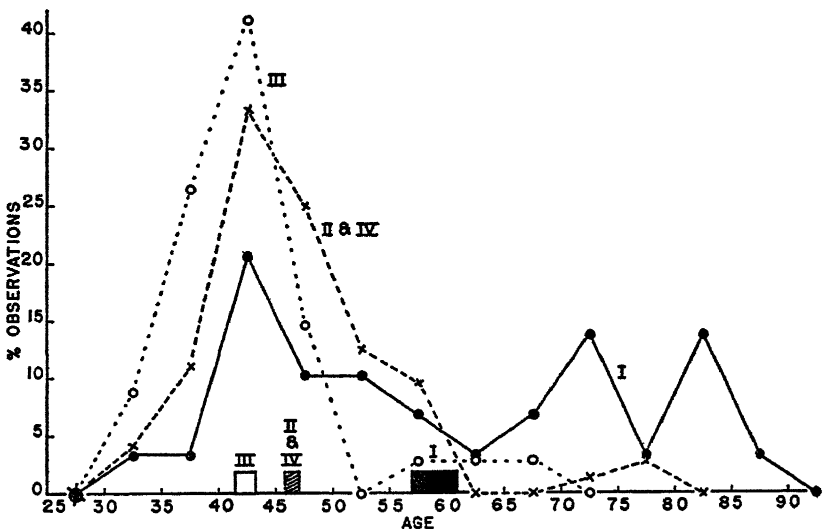

Fig. 2 Frequency polygons of age of opening of vagina. 'McCollum' diet. I = rats kept in complete darkness; II and IV = rats exposed to near and far infra red radiation; III = rats exposed to visible radiation.

difference due to diet in these two groups is not significant. A marked delay is seen in the two groups deprived of light (room I) and also a significant difference between these two groups due to diet, the mean for the 'McCollum' group being 11 days later than that for the Sherman B group. The age for the two groups receiving infra red radiation (rooms II and IV) falls intermediate between groups in rooms I and III, and some effect of diet is evident, the mean for the 'McCollum' group being 3 days later than that for the Sherman B group.

In figures 3, 4 and 5 we have represented, on an upright line, the oestrous history of each female rat from the age of opening of the vagina to 120 days. A similar figure for the groups in rooms II and IV fed the Sherman B diet is omitted for lack of space, but the essential data are given in table 2.

TABLE 1

Age of opening of the vagina

ROOM	SHERMAN B DIET	'MCCOLLUM' DIET
	days P.E.	*days P.E.*
I	48.3±0.92	59.3±2.06
II and IV	44.0±0.75	46.8±0.70
III	41.8±0.65	42.7±0.90

In figures 3, 4 and 5 a circle marks the age of opening of the vagina. An open circle indicates that the rat was not in oestrus at the time of opening, a solid circle in oestrus at time of opening. The subsequent occurrence of oestrus is marked, on the upright line, by a solid circle. In figure 3 two generations in each group are given, separated from each other by a space; in figures 4 and 5 three generations are similarly separated.

Table 2 summarizes some of the data from figures 3, 4 and 5. It gives fairly clear-cut evidence that radiations both in the near infra red and visible regions play a part in the occurrence of the first oestrus. This, in rooms II and III, appears coincident with the opening of the vagina in 80–90% of the animals, whereas the percentages for rooms I and IV are definitely

lower. The presence of fluorine in the diet of the 'McCollum' group does not seem to have affected this result.

Table 2 shows a mean lengthening of the cycle of 1.6 days in the rats in room I fed the Sherman B diet as compared with the group in room III on the same diet. According to the χ^2

TABLE 2

Occurrence of oestrus at time, or within 2 days, of opening of vagina and mean length of cycles

ROOM	SHERMAN B DIET				'MCCOLLUM' DIET			
	In oestrus at time of opening	Mean length of cycle	S.D.	Coefficient of variability	In oestrus at time of opening	Mean length of cycle	S.D.	Coefficient of variability
	%	*days*			%	*days*		
I	45.0	7.3	4.7	53.1	52.2	6.1	3.1	46.2
II	81.8	5.6	..	...	90.0	5.3	..	...
III	80.0	5.7	3.1	53.1	80.0	5.7	2.7	44.0
IV	64.3	6.5	..	...	56.0	6.5	..	...

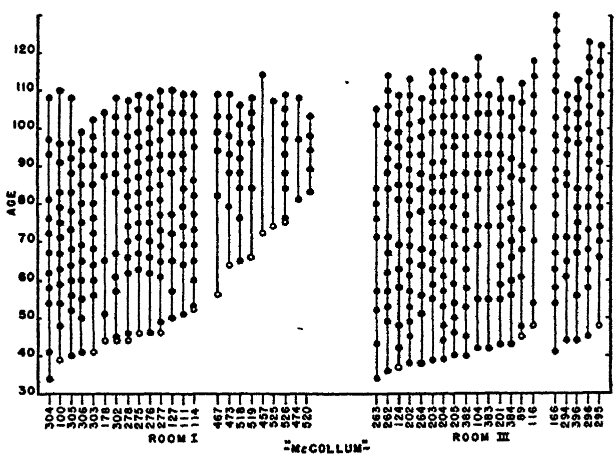

Fig. 3 Oestrous history of individual female rats. 'McCollum' diet. Room I—rats kept in complete darkness. Room III—rats exposed to visible radiation.

method of analysis this result gave a probability of 1 chance in 10,000 in the Sherman B group, and 40 chances in 100 in the 'McCollum' group that the longer cycles observed arose as a matter of chance. We can conclude therefore that in this set of observations the lengthening of the cycles in room I of

rats on the Sherman B diet was a definite finding. There was no significant lengthening of cycles in room I of rats on the 'McCollum' diet. The apparent discrepancy here is possibly explained by the better nutritional value of the 'McCollum' diet despite its contamination.

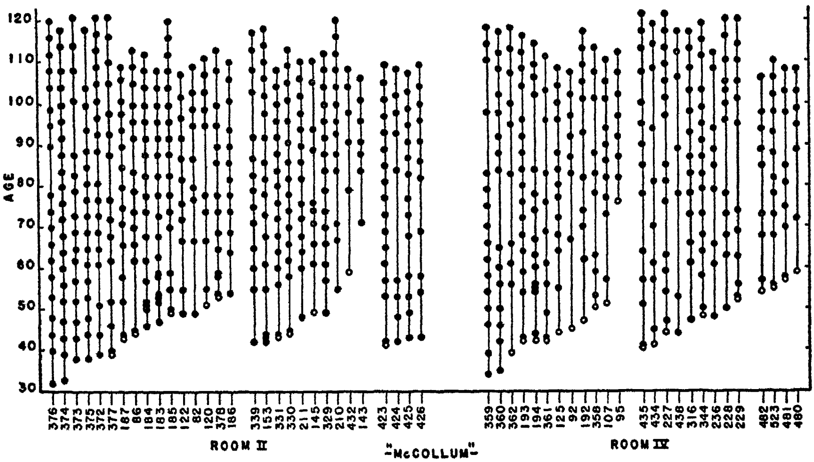

Fig. 4 Oestrous history of individual female rats. 'McCollum' diet. Room II—rats exposed to near infra red radiation. Room IV—rats exposed to far infra red radiaton.

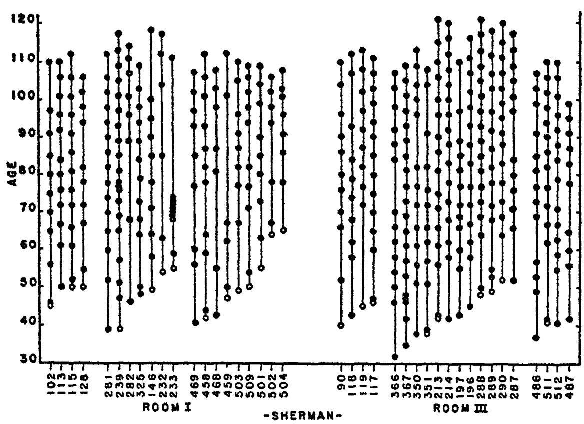

Fig. 5 Oestrous history of individual female rats. Sherman B diet. Room I—rats kept in complete darkness. Room III—rats exposed to visible radiation.

Owing however to the many factors which might produce variability in results, this observation would have to be confirmed. It is evident by inspection of figures 3, 4 and 5 that lengthening of the cycles, in individual rats, occurred at times in all the groups. It is also seen that more rats were affected in this way in room I, and more on the Sherman B than the 'McCollum' diet.

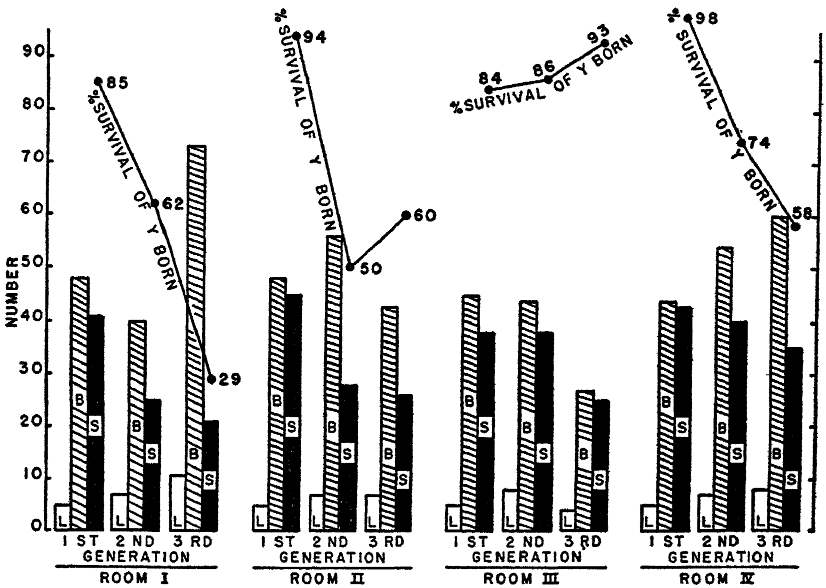

Fig. 6 Birth and survival of young. Sherman B diet. L—number of litters; B—number of young born; S—number of young that survived.

2. Birth and survival of young

(*a*) *Sherman B groups, three generations.* Results are given in figure 6 where the number of litters (L), the total number of young born (B) and the number survived (S) are represented for each generation in each room. The percentage survival of the number of young born in each generation is also indicated.

The percentage survival of young born is normal in all the groups in the first generation. These young were the offspring

of the original pregnant females sent to us from The Wistar Institute.

In room I the failure to survive falls from 62% to 29% in the second and third generations. In room III a normal survival is maintained through three generations. In rooms II and IV the survival in the second and third generations is not normal but superior to results obtained in room I.

It would seem from this that radiation plays a definite role in promoting normal survival of young in rats—that the most effective radiations for this effect are in the visible regions, but that near and far infra red radiation cannot be considered as being without effect.

(*b*) *'McCollum' groups.* The presence of fluorine in the 'McCollum' groups of rats made our results on birth and survival of young inconclusive. The original young of the first generation were reared during lactation by mothers fed the Sherman B diet. By the time they were ready for mating the toxic effect of fluorine was evident and there was only a 50–63% survival of young born in all the groups. No third generation of young was obtained in room I, though a third generation was obtained in rooms II, III and IV. The survival of these radiated young was only from 35–46% of the number born. These results suggest some toxic effect of fluorine transmitted through the milk, but, as in our other results, an effect more marked in the non-irradiated group.

DISCUSSION OF RESULTS

Effect of darkness versus radiation

(*a*) *On rats suffering from fluorosis.* The literature on this subject is scanty. Chaneles ('29) has stated that teeth deformities due to fluorosis in a group of rats which received radiation from a Hanau lamp were less marked than in a similar group which were not irradiated. Smith and Smith ('32) observed that in human teeth, the deposition of pigment, a secondary phenomenon due to fluorosis, occurs on the lip line, or the part of the tooth exposed to light.

It seems to us that the problem of the effect of light on fluorosis merits further study. It is significant, in our results, that the marked delay in the opening of the vagina seen in the second generation of 'McCollum' rats in room I was not apparent in a completely comparable group in room III (fig. 3). Furthermore this delay was not seen in thirty rats from room II, or in twenty-four from room IV (fig. 4), under identical dietary conditions. It is evident from the frequency polygons (fig. 2) that the one for room I includes two sets of observations, the second being a later generation, but that, since all generations are included in polygons III, and II and IV, an effect with succeeding generations is only seen in the group deprived of radiation.

In the teeth of these rats (Hodge, Luce-Clausen and Brown, '39) a lack of pigment in those deprived of light was observed at an earlier date than in the teeth of those exposed to visible radiation. The growth curves (Luce-Clausen and Brown, '39) showed a marked failure of growth of the rats kept in darkness which was not apparent in radiated rats fed the same contaminated diet.

It would seem that all these results point to a combined effect of darkness plus fluorosis on the animals deprived of light. Also that radiation compensates to a demonstrable extent for the full effect of fluorosis as seen in the dark-room animals.

A delay in the onset of maturity in female rats deprived of light appears to be a definite finding. A recent paper by Fiske ('39) suggests that the balance of the two gonadotropic hormones in rats is changed under varying conditions of light. Fiske also observed delay in the onset of maturity in rats kept in darkness. The prolonged periods of oestrus in rats kept under continuous exposure to light which she observed were not apparent in our animals kept under 12-hour alternate exposures to light and darkness. This agrees with the findings of Browman ('37).

We have some evidence that the cycles in female rats in room I were lengthened, and very definite evidence that with

succeeding generations the young born in this room did not survive normally. This failure to survive in young born in the dark increased in the second over the first, and in the third over the second generations. It appeared to be due to some failure in lactation. The young born were normal in birth weight, appeared to be active and healthy, but many died before the fifth day. The mothers, in many instances, appeared nervous, did not make good nests, and showed no inclination to take care of the young. This result again suggests decreased pituitary activity with failure to produce the lactogenic hormone.

The powerful effect of pituitary hormones upon growth and reproductive processes is well known (Evans and Long, '21; Evans and Simpson, '29, '31). It is tempting, from some of our studies, to add radiation, operating through the anterior pituitary in the rat, to the complicated chain of reproductive events. We can only, however, regard this idea as an avenue of approach for future work.

Radiations responsible for the effects described. Radiations throughout the visible and infra red regions of the spectrum have been shown to be effective in promoting growth, early opening of the vagina, early onset of oestrus, and normal survival of young born, in normal animals.

Whatever the biological explanation of these results may be, we have to consider certain possibilities with regard to radiation. Are all the radiations in the spectrum equally effective, and, if so, cannot we regard the effect of radiant energy on the rat, in the spectral regions studied, as simply one of heat with possible resulting catalysis of many chemical reactions in the body? This, at first sight, would appear to be a likely explanation, and is supported by the identical results obtained in rooms II and IV. In these two rooms the rats were exposed to equal energy equivalents, 0.1 gm. cal. per $cm.^2$ per min. (Jones and Tuttle, '39). In room III, however, the picture is different. If we discount secondary radiations which resulted from time to time from heat, the actual amount of energy in the visible was roughly one-third that obtained in rooms II and IV (0.03 gm. cal. per. $cm.^2$ per min.). If we include the

secondary effect of heat, the total energy in room III still falls below that of rooms II and IV (0.08 gm. cal. per cm.^{2} per min.), so that with less energy we get more effect. These results therefore show a peak of maximum efficiency here which seems to be independent of total energy, and obviously referable to illumination. In room III the illumination was 300 foot candles (Jones and Tuttle, '39).

It was surprising to us that any effects at all could be demonstrated in room IV, where the only radiations present were long wave-length infra red. We should have expected results in this room to approximate those in room I, instead of which, in practically every case, they were similar to those in room II. For this reason an effect of radiation was apparent, and was probably only brought out by the efforts made to eliminate all known sources of radiation from room I, and to keep all four rooms at the same air temperature.

It would be very desirable to sub-divide the visible region further, and find out the region of maximum effect. At the time these experiments were planned there was no satisfactory way of doing this, since the light sources available did not emit radiations of sufficient intensity in certain regions, for example, the blue.

CONCLUSIONS

1. The age of opening of the vagina and the onset of first oestrus in female rats is delayed when rats are bred in complete darkness. Radiations in the visible region of the spectrum promote an age of opening of the vagina which is earlier than normal for rats kept under our experimental conditions. Radiations in the near and far infra red promote an age of opening of the vagina similar to that obtained under ordinary conditions of laboratory light here.

2. Radiation plays a definite part in promoting the normal survival of young born in succeeding generations of rats reared under controlled conditions of light. The most effective radiations are in the visible, but radiations in near and far infra red are not without effect.

3. Visible radiation, and, to a lesser extent, infra red radiation, compensate, to a demonstrable effect, for a delay in the

age of opening of the vagina, which increases with succeeding generations, in rats suffering from chronic fluorosis and deprived of light.

4. Radiation, throughout the visible and infra red spectrum, plays a part in reproductive processes in the rat. In the near and far infra red equal results have been demonstrated, using equal energy equivalents in these two regions. Using in the visible region one-third the energy of that used in the infra red, the best results were obtained.

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ADAPTATION TO A LOW CALCIUM INTAKE IN REFERENCE TO THE CALCIUM REQUIREMENTS OF A TROPICAL POPULATION

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ONE FIGURE

(Received for publication April 25, 1939)

Much has been written concerning the calcium requirements of children and adults. Most of the work which throws light upon this matter has been done in Europe and America, and the conclusions which have been drawn are such that they do not appear to be applicable to the conditions in many tropical countries. Sherman ('37) sets the standard allowance for maintenance of the adult at 0.68 gm. and 1 gm. for the growing child. He also states, "It is doubtless better for family food supplies to furnish at least 1 gm. per capita per day."

Leitch ('37) considered the calcium requirements for children and adolescents to range from 0.9 gm. between the ages of 5 and 9, to 1.9 gm. between the ages of 15 and 16 years, and thereafter to decrease gradually to 0.55 gm. at the age of 24 years.

The diets of many tropical races consist of cereals, pulses, roots, vegetables and vegetable oils; milk and eggs seldom appear. Our studies are concerned with Ceylon, where the majority of the poorer classes augment such diets with a little fish. A typical diet of the laboring classes with a value of about 3000 calories, which does not include sprats, contains 0.3 gm. calcium daily. But many of the laboring class Ceylonese take sprats two or three times a week, and this increases the amount of calcium to 0.6 gm. (Nicholls, '37).

The daily diet of the average adult Ceylonese has a value of about 2400 calories and children of 5 years of age consume diets of a value of about 900 calories and those of 10 years of age of about 1800 calories. The amount of calcium in the diets of the children of these ages is a little less than 0.2 and 0.4 gm. respectively. Children are weaned to these diets and the great majority of them never receive milk. The records of one survey of 4221 daily diets of young school children showed that only 251 had received milk (Nicholls, '36).

However deficient such quantities of calcium may appear, the fact that the majority of children become adults shows that they make shift to grow on these quantities. Hence, the question of individual and racial metabolic adaptation arises.

METABOLIC ADAPTATION TO LOW CALCIUM INTAKE

Urinary calcium

Assuming for the sake of argument that there is good absorption of calcium from the alimentary tract whether the total intake is much below or equal to optimum requirements, then if a metabolic adaptation is to take place, it must be in the amount excreted. Sixty-two samples of urine of boys of the laboring classes and an equal number from upper-class boys of the Royal College were analyzed for calcium content. The records of diets of the latter (Nicholls, '37) show that they receive about 1 gm. calcium daily. Table 1 gives the results.

The percentages of table 1 are presented in figure 1, which shows the marked contrast in the amount of urinary calcium in these two groups of boys of different social status.

Calcium balances in growing children

Calcium balances were carried out with four children of the laboring classes. They were given diets similar to those they had received in their homes. The diets were duplicated, one being reserved for calcium analysis. These determinations were made for a period of 3 days for three children, and of

TABLE 1

The calcium content of the urine of upper and lower class boys in Ceylon

AMOUNT OF CALCIUM IN 100 CC. OF URINE	ROYAL COLLEGE		LABORING CLASS	
gm.	*number*	*%*	*number*	*%*
0.0010–0.0030	7	11.3	36	58.1
0.0030–0.0050	11	17.7	19	30.6
0.0050–0.0070	13	21.0	1	1.6
0.0070–0.0090	9	14.5	0	0
0.0090–0.0110	5	8.1	3	4.8
0.0110–0.0130	3	4.8	1	1.6
0.0130–0.0150	3	4.8	0	...
0.0150–0.0170	3	4.8	0	...
0.0170–0.0190	3	4.8	0	...
0.0190 and above	5	8.2	2	3.3
Average amount of calcium in 100 cc. of urine (gm.)	0.0096		0.0041	

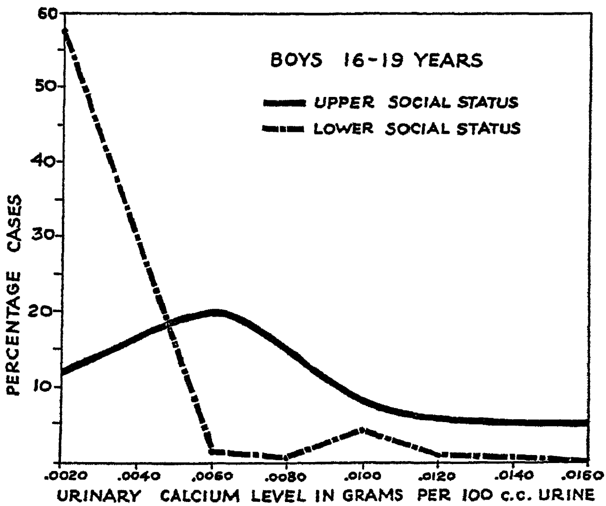

Figure 1

9 days for a fourth child (no. 2). All the children had positive calcium balances, and those on low calcium intakes of about 0.2 gm. a day were able to absorb and retain from 34% to 89% of the calcium (table 2). Child no. 2 for the first 3 days of the experiment had a total daily intake of 0.07 gm. of calcium and retained 0.05 gm. of it.

These children obtain all but a very small amount of vitamin D through the direct action of the sun's rays on their skins and these high retention rates indicate that they receive in this manner an ample quantity of vitamin D.

Table 2 gives the details of these experiments in calcium balances.

TABLE 2

Calcium balances in children

NO. OF CHILD	WEIGHT OF CHILD	AGE AND SEX	PERIOD	DAILY TOTAL INTAKE OF Ca	DAILY TOTAL URINARY EXCRETION OF Ca	DAILY TOTAL FAECAL EXCRETION OF Ca	TOTAL DAILY EXCRETION	DAILY RETENTION OF Ca	Ca RETAINED
	kg.		*days*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	%
1	16.6	7 F	3	0.2454	0.0287	0.1352	0.1639	0.0815	34
2	17.3	7 M	3	0.0703	0.0035	0.0154	0.0189	0.0514	73
2	17.3	7 M	3	0.2225	0.0066	0.0472	0.0538	0.1687	87
2	17.3	7 M	3	0.2196	0.0049	0.0452	0.0501	0.1695	89
3	10.9	4 M	3	0.2051	0.0251	0.0554	0.0805	0.1246	71
4	12.5	4 M	3	0.1833	0.0240	0.0960	0.120	0.0633	41

THE AMOUNT OF CALCIUM IN THE BODIES OF CEYLONESE AT DIFFERENT AGES

The amount of calcium in the body is approximately 25%[1] of the dry weight of the bones of the skeleton, plus 10 to 12 mg. per 100 gm. of the blood and soft tissues of the body. The latter is almost negligible as it amounts to little more than 1% of the total calcium of the skeletal structures.

Many skeletons of children and adults have been collected in the Anatomy School of the Ceylon Medical College. In all cases the ages have been recorded, and in most cases the heights and weights of the cadavers of the children are known.

[1] Bone ash having a composition of 6 $Ca_3(PO_4)_2 \cdot Ca(OH)_2$ is about 70% of the dried bone, which is approximately 25% calcium. Analysis of six samples of dried bone ranged from 22.8–24.9 with an average of 24% calcium.

Prof. W. C. O. Hill kindly allowed us to take the weights and measurements which we required of these skeletons; and in a few cases he permitted the removal of samples of bones for analysis.

Calcium in the skeletons of children

The soft tissues had been removed soon after death and the skeletons cleaned but the bones remained articulated with the ligaments, cartilages, intervertebral discs and epiphyses attached. Each skeleton was weighed and placed in an oven at 100°C. for 6 hours and the dry weight taken. The loss of weight varied from 9.6 to 11.8%. Ten per cent was deducted

TABLE 3

Estimated calcium in children

AGE AND SEX	RACE	BODY WEIGHT	DRY WEIGHT OF ARTICULATED SKELETON	ESTIMATED WEIGHT OF Ca IN SKELETON	
years		*kg.*	*gm.*	*gm.*	*gm./kg.*
3½ F	Sinhalese	13.20	640	144	10.9
4½ F	Sinhalese	14.12	680	155	10.9
5 M	Sinhalese	17.31	824	206	11.9
6 F	Tamil	16.15	770	192	11.8
8 F	Sinhalese	18.80	937	234	12.4
11 M	Sinhalese	23.20	1247	312	13.4

from the weight of each skeleton to allow for the ligaments, cartilages, etc., and the amount of calcium in each skeleton was calculated as 25% of the skeleton after this deduction.

Table 3 gives the details for six of these skeletons and the last column gives the weight of calcium in grams per kilogram body weight.

Calcium in the skeletons of adults

Fifteen skeletons from males and fourteen from females were weighed, and the femurs weighed and measured. Ten samples of bones with contained marrow were taken from these skeletons. They were dried for 3 hours at 105°C. and lost between 8.9 to 10.1% in weight, the average loss being 9.5%. The ash ranged from 64 to 68.4% of the dried samples.

The dried weight of the skeletons was computed by deducting 9.5% for moisture from the actual weight of the bones. The height in life of the person from which the skeleton came has been calculated from the length of the femur by using Pearson's formula. And from tables of the average weight for height for males and females of the various races in Ceylon at different ages, the probable weight of the person from which each skeleton came has been calculated. The average height of the males by these calculations was 164.5 cm. (5 feet, $4\frac{3}{4}$ inches). The average weight for this height is approximately 51 kg.; this gives the amount of calcium per kilogram body weight as 16.5 gm.

The average height of the females by these calculations was 151.6 cm. (4 feet, $11\frac{3}{4}$ inches). The average weight for this height is approximately 41 kg. This gives the amount of calcium per kilogram body weight as 15.2 gm.

The details of these computations are given in table 4 for males and table 5 for females.

Leitch ('37) has calculated the amount of calcium in the body as 30 gm. per kilogram at the age of 5 years, and as 36 gm. from the age of 12 years, 36 gm. per kilogram also being the figure for adults. These figures are more than double the averages obtained by the methods here described; and obviously the amounts of calcium required for growth at different ages, if calculated on the basis of the figures given here will be greatly at variance with the amounts calculated by her figures.

There are no complete European skeletons available in Ceylon for our purposes, but we obtained one femur of a European male and another femur from a European female. We found that the weight of the femur of the skeletons studied by us had a fairly constant relationship to the weight of the entire skeleton, the average being 8.7% for both males and females (column 5, tables 4 and 5). We calculated the entire weights of the skeletons of the Europeans from which the two femurs had come by using this figure. Our calculations are given in the last lines of tables 4 and 5.

TABLE 4

The relation of femur length to size and calcium content of the skeleton: males of India and Ceylon

AGE	RACE	WEIGHT OF SKELETON	WEIGHT OF FEMUR	WEIGHT OF FEMUR IN % OF SKELETON	LENGTH OF FEMUR	ESTIMATED HEIGHT (PEARSON)	ESTIMATED WEIGHT	Ca IN SKELETON	CALCIUM PER KILOGRAM BODY WEIGHT
years		*gm.*	*gm.*		*mm.*	*cm.*	*kg.*	*gm.*	*gm./kg.*
20	Sinhalese	3460	300	8.6	441	164.6	..	783	...
19	Sinhalese	3420	306	8.9	441	164.6	..	774	...
24	Sinhalese	4080	354	8.2	439	164.2	..	923	...
20	Sinhalese	3602	336	9.3	438	163.0	..	815	...
50	Sinhalese	4290	374	8.7	452	166.6	..	971	...
30	Malayalee	4090	350	8.2	448	166.0	..	925	...
50	Sinhalese	3870	337	8.7	439	164.2	..	876	...
30	Sinhalese	3740	304	8.1	436	162.3	..	845	...
21	Sinhalese	3927	349	8.8	443	165.0	..	890	...
30	Indian Tamil	3660	314	8.5	446	165.5	..	828	...
28	Indian Tamil	3900	345	8.8	443	165.0	..	883	...
50	Indian Tamil	3134	292	9.3	452	166.6	..	684	...
25	Indian Tamil	2723	241	8.8	426	161.7	..	616	...
40	Indian Tamil	3477	350	10.0	429	162.3	..	788	...
30	Ceylon Tamil	4482	382	8.4	452	166.6	..	1014	...
	Average	3724	329	8.8	442	164.5	51	841	16.5
	European	5295	452	..	460	167.8	65	1198	18.4

TABLE 5

The relation of femur length to size and calcium content of the skeleton: females of India and Ceylon

AGE	RACE	WEIGHT OF SKELETON	WEIGHT OF FEMUR	WEIGHT OF FEMUR IN % OF SKELETON	LENGTH OF FEMUR	ESTIMATED HEIGHT (PEARSON)	ESTIMATED BODY WEIGHT	Ca IN SKELETON	Ca PER KILOGRAM BODY WEIGHT
years		*gm.*	*gm.*		*mm.*	*cm.*	*kg.*	*gm.*	*gm.*
35	Sinhalese	3420	282	8.2	439	158.2	..	774	...
30	Sinhalese	3147	262	8.2	418	154.1	..	712	...
18	Sinhalese	2296	271	10.8	391	148.8	..	520	...
20	Sinhalese	2634	239	9.1	417	153.9	..	596	...
25	Sinhalese	2320	206	8.8	398	150.2	..	525	...
35	Sinhalese	2410	193	8.0	386	147.9	..	545	...
21	Sinhalese	2560	210	8.2	386	147.9	..	579	...
Aged	Sinhalese	2925	249	8.5	441	158.6	..	662	...
28	Sinhalese	3590	340	9.4	413	153.1	..	817	...
Aged	Tamil	2060	187	7.9	423	155.1	..	466	...
30	Tamil	2340	188	8.0	368	144.4	..	530	...
28	Tamil	2880	267	9.2	393	149.2	..	652	...
Aged	Tamil	2410	212	8.7	415	153.5	..	542	...
45	Tamil	3750	335	8.7	433	157.0	..	848	...
	Average	2767	246	8.7	409	151.6	41	626	15.2
	European	2850	248	..	437	157.8	48	645	13.4

The skeleton showed that the European woman was of light build, and we have guessed her weight to have been about 7½ stone (48 kg.). The skeleton of the male was of robust build, and 10¼ stone (65 kg.) is probably a conservative estimate. Although the method of estimating the amount of calcium in these European skeletons cannot be considered very satisfactory, yet it is sufficient to indicate that the amount of calcium per kilogram body weight is not markedly different in Ceylonese and Europeans.

Increase in the calcium in the body during the years of growth

The ultimate aim is to determine the calcium requirements for use in the prescription of diets; and as there are individual variations, within small limits, of the amount of calcium per kilogram body weight only round figures may be used, and these should be a little higher than the estimated average. The increase in the calcium in the body during the years of growth can be approximately determined by using the figures given in tables 3, 4 and 5 of the amount of calcium per kilogram body weight of children and adults. We base the requirements on 12 gm. of calcium per kilogram body weight for a child aged 5, and neglecting variations in growth, and allowing an increase of 0.5 gm. a year the figure 17 gm. per kilogram is reached at the age of 15 years.

The weights at different ages of children and adolescents of the various social classes have been published (Nicholls, '36).

The figures for males of the upper social status are for the youths and boys of the Royal College and its preparatory school; the figures for males of the lower social status are for boys of the free schools and others of the laboring classes. The former are considerably heavier at all ages. Table 6 gives the average weights of these boys at different ages, and from the amount of calcium per kilogram of body weight, the total amount of calcium in the body is calculated. From the increased amount of calcium in the body each year, the average daily retention throughout the previous year has been determined. The columns headed B.M. in table 6 are the weights

TABLE 6

Increase in the calcium in the body of males between the ages of 5 and 17 years

AGE	BODY WEIGHT			CALCIUM PER KILOGRAM BODY WEIGHT	CALCIUM IN BODY			INCREASE OF CALCIUM IN BODY SINCE PREVIOUS YEAR			DAILY RETENTION OF CALCIUM			CALCIUM REQUIREMENT;[4] U.S.S.
	L.S.S.[1]	U.S.S.[2]	B.M.[3]		L.S.S.	U.S.S.	B.M.	L.S.S.	U.S.S.	B.M.	L.S.S.	U.S.S.	B.M.	
years	*kg.*	*kg.*	*kg.*	*gm./kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
5	15.0	17.0	18.1	12	180	204	217	..	..	..	....	....	....	...
6	16.2	18.8	20.2	12.5	202	236	253	22	32	36	0.060	0.087	0.101	0.35
7	17.3	20.4	22.6	13	225	265	294	23	29	41	0.063	0.079	0.112	0.35
8	19.0	23.9	25.0	13.5	257	323	338	32	58	44	0.087	0.161	0.120	0.56
9	20.2	25.2	27.4	14	283	353	384	26	30	46	0.071	0.082	0.126	0.41
10	22.3	27.9	30.6	14.5	323	395	437	40	42	53	0.109	0.115	0.145	0.51
11	23.1	29.8	32.6	15	346	447	489	23	52	52	0.063	0.142	0.142	0.68
12	25.4	33.6	34.8	15.5	394	515	539	48	68	50	0.131	0.186	0.137	0.70
13	26.8	36.4	37.4	16	429	582	598	35	59	59	0.095	0.161	0.161	0.68
14	30.5	44.5	41.7	16.5	503	734	688	74	152	90	0.202	0.416	0.244	1.30
15	34.9	47.8	46.6	17	593	813	792	90	79	104	0.244	0.216	0.285	0.91
16	38.1	50.8	54.0	17	648	844	918	55	31	126	0.153	0.084	0.345	0.66
17	43.6	54.7	59.4	17	741	930	1010	93	86	92	0.254	0.238	0.249	1.10

[1] L.S.S.—males of lower social status.
[2] U.S.S.—males of upper social status.
[3] B.M.—British males.
[4] Calculated in grams as 2 × retention + 10 mg./kg.

and similar calculations for British males on the assumption that the average amount of calcium per kilogram body weight is more or less the same for any particular age group of males of any country.

Table 7 gives the figures for females. The two social statuses in this case are the girls of the primary and secondary schools. It was not possible to obtain the weights of girls of the highest social status.

It will be seen from table 6 that the daily retention of calcium by the L.S.S. males ranges from 0.06 gm. between the ages of 5 and 6 years to 0.254 gm. between the ages of 15 and 16 years. In the case of the U.S.S. males this retention ranges from 0.079 gm. between the ages of 6 and 7 years to 0.416 gm. between the ages of 13 and 14 years. The retention for British males is from 0.101 gm. between the ages of 5 and 6 years to 0.345 gm. between the ages of 15 and 16 years.

Table 7 shows that the retention rates are somewhat lower for females.

Leitch suggests that the daily calcium requirements should be double the daily retention rate plus 10 mg. per kilogram body weight. These suggested requirements are given in the last column of tables 6 and 7 and are calculated for the U.S.S. boys and the S.S. girls.

DISCUSSION

There are two factors among many which must be considered in relation to the question of calcium requirements. Firstly, growth does not take place at a uniform rate; the same applies to many, if not all metabolic processes. Periods of relatively great activity are succeeded by periods of less activity. This is shown for growth in any tables of the heights and weights of children at various ages. The Ceylon tables, from which the weights in tables 6 and 7 have been produced were drawn up from the measurements and weights of over 12,000 Ceylonese children; they show that the primary school girls have a rapid rate of growth between the ages of 7 and 8 followed by a very slow rate between the ages of 8 to 9; a

TABLE 7

Increase in the calcium in the body of females between the ages of 5 and 17 years

AGE	BODY WEIGHT			Ca PER KILOGRAM BODY WEIGHT	CALCIUM IN BODY			INCREASE OF CALCIUM IN BODY FROM PREVIOUS YEAR			DAILY RETENTION OF CALCIUM			CALCIUM REQUIREMENT:[4] S.S.G.
	P.S.G.[1]	S.S.G.[2]	B.G.[3]		P.S.G.	S.S.G.	B.G.	P.S.G.	S.S.G.	B.G.	P.S.G.	S.S.G.	B.G.	
years	*kg.*	*kg.*	*kg.*	*gm./kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
5	15.0	16.6	17.7	12	180	199	212	..	..	..	....	....	....	....
6	15.7	17.5	19.0	12.5	196	219	237	16	20	16	0.044	0.055	0.044	0.285
7	17.0	19.0	21.5	13	221	247	274	25	28	37	0.068	0.076	0.101	0.342
8	19.0	20.3	23.6	13.5	256	274	319	35	27	45	0.096	0.074	0.123	0.351
9	20.6	22.1	25.2	14	288	308	353	32	34	34	0.087	0.092	0.092	0.405
10	22.5	24.3	28.1	14.5	326	352	407	38	44	54	0.104	0.121	0.148	0.485
11	23.9	27.9	30.8	15	359	419	462	33	67	55	0.090	0.184	0.151	0.647
12	26.5	30.2	34.7	15.5	411	468	538	52	49	76	0.142	0.134	0.208	0.570
13	29.4	34.7	39.5	16	470	555	632	59	87	94	0.162	0.238	0.258	0.823
14	32.0	37.6	43.9	16	512	602	702	42	47	70	0.115	0.129	0.191	0.634
15	36.9	40.5	48.2	16	590	648	771	78	46	69	0.213	0.126	0.189	0.657
16	38.2	42.3	51.3	16	611	677	821	21	29	50	0.057	0.078	0.137	0.579
17	39.2	43.3	52.4	16	627	693	838	16	16	17	0.043	0.043	0.046	0.519

[1] P.S.G.—primary school girls.

[2] S.S.G.—secondary school girls.

[3] B.G.—British girls.

[4] Calculated in grams as 2 × retention + 10 mg./kg.

similar state of affairs occurs between 9 to 10 and 10 to 11. The secondary school girls are a year ahead, i.e., the years of rapid growth are 8 to 9 and 10 to 11, with both periods being followed by a slowing down of growth; similar pulsations of growth are shown in the tables for the boys. Even in tables drawn up from the statistics of very large numbers of children similar pulsations can be seen, although it might be expected that when dealing with large numbers the irregularities in the growth curve would be smoothed out, because some children are backward and others forward in growth.

It appears that when growth has been affected by ill health, or by poor diets, compensations take place. For instance, rapid rates of growth of the higher class Ceylon boys cease after the age of 17 years, but the poorest class boys have periods of rapid growth between the ages of 17 and 18 years; similarly the secondary school girls have no periods of rapid growth after the age of 15 years, whereas the free school girls have periods of moderately rapid growth after this age. But there is no complete compensation, because the averages for the poorer classes are in all cases far below the averages for the better classes.

The amount of calcium retained by growing children must be of the same order as the rate of growth. Not only are there long periods of high retention of a year or more, followed by long periods of low retention, but there are lesser pulsations in calcium retention extending over a number of days. The percentages of retention of the calcium intake, as shown in table 2 varied from 34% to 89%. Other observers have found great variations in the amount of calcium retained. Lusk ('28) gives an example of two children for whom calcium balances were determined for 10 days; K aged $5\frac{1}{2}$ years had a daily intake of 0.91 gm. of calcium and retained 0.039 or about 4% of the intake; the second child N aged $7\frac{3}{4}$ had a daily intake of 1.3 gm. and the daily retention was 0.478 [2] or about 36% of the intake. The child K was in a period of low calcium intake

[2] The figures of Lusk's table on page 573 are given in terms of CaO, and have been reduced to equivalents for Ca.

which could not continue or the child would become a dwarf, nor could the high calcium intake of the second child continue for long because the average daily amount of calcium required between the ages of 8 and 20 to produce a well-grown skeleton is less than 0.2 gm.

In the adult, calcium is continually being withdrawn from and returned to the skeleton, hence negative balances followed by positive balances must be a normal state of calcium metabolism.

The second factor is adaptation, whether individual or racial. If negative balances exceed the positive balances over a long period of time in any individual, osteoporosis must eventually supervene. Many races more or less vegetarian in food habits, especially in lands with long dry seasons, must make shift on a low calcium intake. In the dry zones of Ceylon leafy vegetables are not obtainable for many months, and as the inhabitants do not take milk or eggs, the diets are very low in calcium; yet there is no evidence of the existence of osteoporosis such as a high incidence of fractures, and the skeletons we have examined appear normal.

Sherman based his estimate of adult calcium requirement on metabolism experiments. When in any individual the intake was followed by retention he considered the requirements of the individual to be the amount of the intake less the amount retained; and when there was a negative balance the requirement was considered to be the amount of the intake plus the difference between the intake and the amount excreted—that is, the loss. Leitch also calculated the calcium requirements on calcium balance experiments. Neither of these authors takes into account the question of adaptations to various intakes. If a person is accustomed to a calcium intake of 0.6 gm. daily the metabolic processes will become adapted to this amount and the negative and positive balances will be more or less equal; but if suddenly his intake is reduced to 0.3 gm. his metabolism will not be adapted to this amount for sometime and consequently this intake will be followed by negative balances. On the other hand, if he has been accustomed to an

intake of only 0.3 gm., on an intake of this amount the negative and positive balances will be more or less equal.

It follows from this argument that, provided the calcium balances of an individual are determined over a sufficiently long period of time, all that such experiments will reveal is the daily amount of calcium the individual is accustomed to take; and calculation from these of optimum requirements cannot be made.

The heights and weights of the Ceylon children of the poorer classes compare unfavorably with those of the children of the upper, and it may be concluded that they are stunted. It may be that calcium deficiency plays a small part, but there is no clinical evidence of this; whereas the majority of the children of the poorer classes show marked signs of vitamin deficiencies, such as phrynoderma, sore mouth, Bitot's spots, xerophthalmia and night blindness at one time or another during the period of their growth, these signs are absent from the children of the upper classes (Nicholls, '34, '35, '36).

Therefore standard allowances may safely be based upon the requirements of the children of the upper classes, because they are well grown. If it is assumed that the amount of calcium per kilogram body weight is more or less the same in British children as in Ceylon children then these allowances should be applicable in Britain.

The calcium requirements calculated by the formula—grams of calcium retained $\times$ 2 + 10 mg. per kilogram body weight —give generous allowances. And the following, in a convenient form for practical use, are the standard allowances as calculated for the Ceylon children of the upper social status as given in the last columns of tables 6 and 7:

For children from age of 1 to 7 years—0.5 gm. of Ca.
For children from age of 7 to 12 years—0.75 gm. of Ca.
For adolescents from age 12 to 20 years—1.00 gm. of Ca.

A sufficiency of any constituent in the diets of healthy individuals is the optimum, nothing is gained by an excess, and as a general principle, dietary excesses should be avoided.

Whether or not a high intake of calcium over a long period is detrimental to health is unknown.

A standard allowance of 0.5 gm. of calcium daily appears to be sufficient for adults, excluding lactating women.

SUMMARY

1. The standard allowances for the daily intake of calcium for children and adults as given by authorities in Europe and America are considered too high.

2. There is evidence of metabolic adaptation to a low calcium intake as shown by: (a) The low amounts of calcium in the urines of those on a low calcium intake, when compared with the amounts in the urines of those on a high calcium intake. (b) The high percentage of calcium retention by growing children on about 0.2 gm. of calcium daily.

3. Many skeletons of Ceylonese children and adults have been weighed, and ten of these have been analyzed for moisture and ash. The amounts of calcium in the skeleton at different ages, and the amounts of calcium per kilogram body weight have been calculated.

The amounts of calcium retained daily by children and adolescents of different ages have been calculated from this.

4. Daily standard allowances for children and adults are suggested.

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THE ROLE OF THE ANTIDERMATOSIS VITAMIN AND A NEW WATER-SOLUBLE GROWTH FACTOR IN THE NUTRITION OF THE MATURE FOWL

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Ringrose, Norris and Heuser ('31) were the first investigators to report the occurrence of dermatosis in chicks. Later Kline, Keenan, Elvehjem and Hart ('32) found that dermatosis, similar to that described by Ringrose and Norris but a more aggravated form, developed in chicks fed a diet that had been heated in a dry atmosphere for 144 hours at 100°C. Both groups of investigators considered that the factor preventing the dermatosis was vitamin G or B_2. Ringrose and Norris ('34) reported that the factor which prevented dermatosis in chicks was a complex consisting of a growth-promoting component, stable to prolonged dry heat treatment, and a dermatosis-preventing component, unstable under these conditions. Lepkovsky and Jukes ('35) also presented evidence that vitamin G consisted of two components, one of which was adsorbed upon fuller's earth while the other remained in the filtrate. Ringrose and Norris ('36 b) confirmed the results of Lepkovsky and Jukes showing that these components of the vitamin G complex are differentially adsorbed upon fuller's earth. The component remaining in the filtrate prevented dermatosis in chicks. It was called the filtrate factor by Lepkovsky and Jukes. These investigators found that the dermatosis-preventing factor was present in the filtrate obtained by treating a water-extract of rice bran with fuller's earth as well as in certain other materials.

Since the discovery of Kline and associates ('32) that the antidermatosis vitamin is destroyed by prolonged dry heat treatment, dermatosis has been frequently produced in the experimental laboratory. No evidence, however, that the mature fowl suffers from a deficiency of this vitamin has yet been reported. Hence, a study of this problem was undertaken, the results of which are presented in this paper. In the course of the study evidence was obtained that the experimental diets were deficient in another factor required for growth and reproduction as well as the antidermatosis vitamin. The results demonstrating this are also presented herein. Since the initiation of the study, Lepkovsky, Taylor, Jukes and Almquist ('38) have reported that the antidermatosis vitamin, under the conditions studied, seems to exercise no function in maintaining normal egg production and hatchability.

EXPERIMENTAL PROCEDURE

White Leghorn chicks and hens were used as experimental subjects in this study. The chicks were the progeny of hens fed in an adequate manner except where otherwise stated. All hens were reared and fed normally and were in full production at the time the experimental work was begun. All chicks and hens were selected for desired qualities at the beginning of each experiment after which they were distributed into groups. The pens in which the chicks and hens were confined were equipped with wire-mesh floors in order to reduce coprophagy to a minimum. In the work on reproduction the males were rotated among the lots frequently in order to rule out differences in breeding capacity. Both chicks and hens were identified by numbered wing or leg bands. The chicks were weighed weekly and the hens by periods of 2 weeks. The significance of the growth differences between the lots of chicks was calculated according to the method of Titus and Hammond ('35). Observations were made at time of weighing for the occurrence and severity of dermatosis in each chick. The degree of dermatosis per lot was then calculated according to the procedure of Ringrose and Norris ('36 a).

The diets used in this study were adapted after the heated diet used by Kline and associates ('32) in producing dermatosis in chicks. In order to render the diets free of the antidermatosis vitamin, the cereal portion and the liver extract (pre-mix) were heated in an oven for 36 hours at 120°C., and the casein was purified by the method of Sharp and DeTomasi.[1] Henceforth, the diets containing the unheated cereals are referred to as diet CA (chick adequate diet) and diet HA (hen adequate diet) and the diets free of the antidermatosis vitamin as diet CD (chick deficient diet) and diet HD (hen deficient diet). Table 1 gives the composition of diets CA and HA.

TABLE 1

Experimental diets

PRE-MIX	PARTS
Yellow corn meal	40.65
Wheat bran	20.00
Wheat flour middlings	20.00
Liver extract	0.35

	CHICK DIET %	HEN DIET %
Pre-mix	79.25	81.00
Purified casein	13.00	7.00
Crude expeller soy bean oil	4.00	5.00
Wheat germ oil	....	0.50
Cod liver oil	0.25	0.50
Pulverized limestone	2.00	3.50
Steamed bone meal	1.00	2.00
Iodized salt	0.50	0.50
Manganese carbonate	....	trace
Protein content	21.00	16.50

The cod liver oil used in these diets was a biologically assayed one, containing 3000 U.S.P. units of vitamin A per gram and 250 A.O.A.C. units of vitamin D per gram. According to Norris ('39), this oil in the amounts used provided quantities of vitamin A and D in excess of the requirements for growth and reproduction. Although vitamin E is stable

[1] Unpublished method by Sharp and DeTomasi of the Department of Dairy Husbandry, Cornell University.

to dry heat treatment, biologically assayed wheat germ oil was added to the hen diets in an amount that provided approximately ½ cc. of oil per hen per day. According to Card, Mitchell and Hamilton ('30), this quantity of wheat germ oil is sufficient to provide for the vitamin E needs of the hens exclusive of that in the cereal portion of the diet. The soy bean oil used in the diets was biologically assayed for vitamin K, using a modification of the method of Almquist and Stokstad ('37). In chicks fed diet CD a level of 4% promoted normal blood-clotting time. This oil was found to prevent chicks from developing nutritional encephalomalacia when fed the diet of Pappenheimer and Goettsch ('31); also it prevented the development of ataxic symptoms in chicks fed the vitamin B_4-deficient diet of Kline, Bird, Elvehjem and Hart ('36). The cod liver oil and wheat germ oil were added to the diets at intervals not greater than 7 days while the soy bean oil was added at the beginning of each experiment.

The deficient diets were found to be adequate in thiamin since the feeding of 75 μg. of thiamin hydrochloride per chick daily did not increase the growth of chicks fed diet CD above that of chicks receiving diet CD alone. Furthermore, polyneuritis was never observed in chicks fed this diet. These results therefore confirm those of Keenan, Kline, Elvehjem and Hart ('35).

Although no evidence has appeared showing that vitamin B_6 is essential in poultry nutrition, the deficient diets were proved to be adequate in this vitamin by means of the bioassay procedure of Bender and Supplee ('37), in which rat growth and the development of acrodynia are used as criteria. Nicotinic acid supplied either orally or parenterally failed to stimulate the growth of chicks fed diet CD above that obtained on diet CD alone. It was also ineffective in preventing or curing the dermatosis which develops in chicks fed this diet in agreement with results previously reported by Mickelsen, Waisman and Elvehjem ('38).

The results of fluorometric determinations by the method of Hodson and Norris ('38) showed that the liver extract here

used contained approximately 800 µg. of riboflavin per gram. This was in agreement with the results of a bioassay with chicks which showed that the liver extract contained approximately 770 µg. of riboflavin per gram. In this same bioassay it was found that the deficient diets contained 330 µg. of riboflavin per 100 gm., an amount in excess of that reported by Norris, Wilgus, Heiman, Ringrose and Heuser ('36) to be necessary for growth and reproduction. It was further found that the riboflavin content of the egg albumen of the eggs of hens fed diet HA and that of the eggs of the hens fed diet HD was approximately the same.

From the results of the bioassays of the deficient diets as well as those of certain of the special ingredients, it is evident that they were adequate in vitamins A, B, B_4, B_6, D, E, G, K, and the antiencephalomalacia factor. The diets were also either adequate in nicotinic acid or this vitamin is not required in poultry nutrition. These conclusions are supported by the failure of any characteristic symptoms of a deficiency of any of these vitamins to develop in the chicks and hens used in subsequent experiments.

Rice bran filtrate[2] prepared according to the method of Lepkovsky and Jukes ('35) was used as the source of the antidermatosis vitamin in this study. It was made from an aqueous extract of rice bran by twice subjecting the extract to fuller's earth adsorption, filtering off the fuller's earth, and concentrating the filtrate under vacuum. The filtrate, when assayed according to the method of Lepkovsky and Jukes ('36), was found to contain 25 units of the antidermatosis vitamin per gram.

Before beginning the work with hens it was felt desirable to determine whether or not diet CD, when supplemented with a sufficient quantity of the rice bran filtrate, would promote the same rate of growth as diet CA and thus be shown to be complete in all factors present in the latter diet, except the antidermatosis vitamin. Graded quantities of the rice bran filtrate were added to diet CD and fed to a corresponding

[2] Obtained from Vitab Products, Inc., Emeryville, California.

number of lots of chicks. One lot fed diet CD alone served as a negative control, and another fed diet CA as a positive control. The chicks were depleted of their reserves of the antidermatosis vitamin for 1 week before being placed upon experiment. The outline of the experiment and a summary of the results are presented in table 2.

Some dermatosis developed in nearly all lots during the first week that the chicks were on experiment as a consequence of the depletion during the previous week. In the negative control lot and the lots receiving 1 and 2% of the rice bran filtrate, the percentage degree of dermatosis gradually increased during the course of the experiment. In the lot receiving 4% of the filtrate, there was no appreciable increase in the degree of dermatosis, while in the lot receiving 8% the degree of dermatosis decreased. In a previous experiment the degree of dermatosis decreased slightly under identical circumstances in the lot receiving 4% of the filtrate. It is evident from these results that 4% of the filtrate supplied a sufficient amount of the antidermatosis vitamin to prevent the development of dermatosis. According to Almquist, Jukes and Newlon ('38) a chick diet containing 90 units of the antidermatosis vitamin per 100 gm. contains a sufficient amount for normal growth.

TABLE 2

Adequacy of diet CD when supplemented with rice bran filtrate (fourteen chicks per lot)

TREATMENT	DEGREE OF DERMATOSIS At 2 weeks	DEGREE OF DERMATOSIS At 5 weeks	MORTALITY AT 5 WEEKS	WEIGHT AT 5 WEEKS	SIGNIFICANCE OF DIFFERENCE
	%	%	%	gm.	't' [1]
Diet CD	6.4	37.6	7.2	89.0 >	2.29
CD + 1% rice bran filtrate	6.4	27.4	7.2	114.2 >	3.61
CD + 2% rice bran filtrate	5.5	19.7	7.2	152.1 >	2.25
CD + 4% rice bran filtrate	2.4	2.8	14.4	184.6 >	2.00
CD + 8% rice bran filtrate	1.6	0.0	7.2	206.0 >	5.44
Diet CA	0.0	0.0	7.2	266.5	

[1] t = 1.99, odds = 19:1; t = 2.64, odds = 99:1.

The growth of the chicks fed diet CD containing 8% of the rice bran filtrate was much less, however, than that attained on diet CA. The difference in growth was strikingly significant. On the other hand, the difference in growth between the lot fed the 8% level of the filtrate and the 4% level was barely significant. From these results it is evident that there is a growth factor lacking in diet CD which is not supplied in adequate amounts by rice bran filtrate and hence is not identical with the antidermatosis vitamin. Since diet CD was shown to be adequately supplied with all the known vitamins except the antidermatosis vitamin, the missing factor cannot be one of them. It is organic in nature, since it is destroyed by dry heat treatment. A preliminary report of these results has been made by Bauernfeind, Schumacher, Hodson, Norris and Heuser ('38).

In an attempt to find sources of the missing factor which could be used to make the deficient diets complete, when supplemented with the antidermatosis vitamin, resort was made to dried liver and liver extract, since Ringrose and Norris ('36 b) showed that a chick diet similar to diet CD when supplemented with dried liver promoted a superior rate of growth in chicks. Whey adsorbate was also used in this experiment, since from information available at this laboratory, it was possible to show that the growth response which Jukes ('37) obtained from this product could not be accounted for by its riboflavin content. The results of this experiment are presented in table 3. They show that these products at the levels used promoted growth which was significantly greater than that obtained in chicks fed diet CD supplemented with rice bran filtrate alone. The growth stimulated by these products was nearly as great as that promoted by diet CA.

In further work it was found that rice bran extract, rice bran adsorbate, dried brewer's yeast, autoclaved brewer's yeast, aqueous extract of brewer's yeast and grass juice promoted an increase in weight above that of the control lot which was strikingly significant in all but two instances. In these instances the growth difference was considerably better than

that required for mere statistical significance. The growth obtained in most instances was approximately equal to that obtained on diet CA.

These results showed that the factor lacking in the deficient diets, when supplemented with the antidermatosis vitamin, is water-soluble, adsorbed on fuller's earth and not destroyed in

TABLE 3

Sources of the new growth factor

(first experiment sixteen chicks per lot; second experiment twelve chicks per lot)

TREATMENT	MORTALITY	WEIGHT AT 5 WEEKS	SIGNIFICANCE OF DIFFERENCE
Experiment 1			
	%	*gm.*	't'[1]
Diet CD + 6% rice bran filtrate (BD)	12.5	203.2	
BD + 300 μg. riboflavin per 100 gm.	6.3	208.3	0.4
BD + 4% whey adsorbate no. 1	5.3	235.0	2.5
BD + 3% liver adsorbate	0.0	239.1	2.8
BD + 0.35% liver extract	12.5	240.4	2.9
Diet CA	6.3	250.4	3.7
Experiment 2			
			't'[2]
Diet CD + 6% rice bran filtrate (BD)	0.0	204.3	
BD + 300 μg. riboflavin per 100 gm.	0.0	208.8	0.4
Diet CD + 6% rice bran extract	0.0	254.1	3.7
BD + 4% rice bran adsorbate	8.3	243.8	2.9
BD + 1% whey adsorbate no. 2	0.0	246.3	3.0
BD + 5% brewer's yeast	0.0	308.5	7.7
BD + 5% autoclaved yeast	0.0	301.2	7.3
BD + aqueous extract of brewer's yeast ≎ 5%	8.3	276.3	4.9
BD + 25% grass juice	16.6	267.8	4.8
Diet CA	0.0	275.1	5.2

[1] First experiment t = 1.9, odds 19: 1; t = 2.6, odds 99: 1.

[2] Second experiment t = 2.0, odds 19: 1; t = 3.6, odds 99: 1.

yeast by autoclaving for 5 hours at 15-pound pressure. By treatment of rice bran extract with fuller's earth the new factor is separated from the antidermatosis vitamin and by prolonged dry heat treatment from riboflavin.

In the experimental work on the role of the antidermatosis vitamin in the nutrition of the mature fowl two experiments

were conducted. In both experiments the hens were fed diet HD alone until the hatchability of their eggs approached zero. The plan of both experiments and the results obtained are presented in table 4. In the first experiment the average hatch of the fertile eggs of the hens fed diet HD varied from 2.5 to 4.5% as compared to 60.8 to 68.8% for the eggs of the hens fed diet HA. When diet HD was supplemented with 5% of rice bran filtrate, a slight improvement in reproduction was

TABLE 4

Effect of the antidermatosis vitamin and the new growth factor upon the nutrition of the mature fowl

TREATMENT AND EXPERIMENTAL PERIOD	HENS PER PEN	LENGTH EXPERIMENTAL PERIOD	AVERAGE WEIGHT	AVERAGE EGGS PER HEN PER WEEK	EGGS SET	HATCH
		Experiment 1				
	no.	*weeks*	*gm.*	*no.*	*no.*	*%*
Diet HA (1)	24	8	1893	3.34	581	68.8
Diet HA (2)	23	6	1934	2.98	351	68.6
Diet HA (3)	21	6	1843	2.57	232	60.8
Diet HD (1)	14	8	1780	3.41	357	4.5
Diet HD (2)	13	6	1696	2.14	145	2.5
Diet HD (3)	6	6	1697	3.73	122	2.9
HD + 5% rice bran filtrate (1)	15	8	1772	3.50	416	12.3
HD + 10% rice bran filtrate (2)	14	6	1694	2.72	193	6.3
HD + 5% whey adsorbate (3)	10	6	1523	2.57	152	3.2
HD + 5% whey abdorbate + 5% rice bran filtrate (3)	11	6	1453	2.33	136	50.6
		Experiment 2				
Diet HA (1)	17	6	1779	3.51	301	54.0
Diet HA (2)	12	5	1662	3.42	188	65.4
Diet HD (1)	17	6	1718	3.29	274	2.5
Diet HD (2)	12	5	1667	4.26	253	6.6
HD + 5% rice bran filtrate (1)	17	6	1732	3.85	330	14.0
HD + alcohol ppt. ≎ 7.5% brewer's yeast (2)	12	5	1719	3.91	217	8.7
HD + alcohol ppt. ≎ 7.5% brewer's yeast + 5% rice bran filtrate (2)	12	5	1676	3.51	195	50.6

obtained. In the next experimental period, however, this improvement was not maintained, as the hatch of the fertile eggs dropped to 6.3%, in spite of the fact that the amount of rice bran filtrate in diet HD was increased to 10%. When diet HD was supplemented with 5% of whey adsorbate, the average hatch of the fertile eggs was no greater than that obtained with the rice bran filtrate. On the other hand, when diet HD was supplemented with both rice bran filtrate and whey absorbate, the average hatch of the fertile eggs was 50.6%, a striking increase above that obtained when either of these supplements was fed alone. It is evident from these results, therefore, that the rice bran filtrate (antidermatosis vitamin) and the whey adsorbate (new factor) are both required for reproduction in the domestic fowl. A preliminary announcement of the role of the antidermatosis vitamin in reproduction was made by Bauernfeind and Norris ('39).

The hens fed diet HD failed to attain as good reproduction or maintain their weight as well as those fed diet HA. Whether these results were due to the fact that more of the new factor is required for reproduction and maintenance of weight than was fed, or whether diet HD is lacking in another factor, or factors, required for these purposes, cannot be determined from the evidence. Since the average egg production per hen per week was approximately the same throughout this experiment, it appears that neither the antidermatosis vitamin nor the new factor, under the conditions of the experiment, is concerned in the maintenance of egg production.

In a repetition of this work the following year using another group of hens, results were obtained which confirmed those of the first experiment. The plan of the second experiment and the results are also presented in table 4. In this experiment the average hatch of the fertile eggs of the hens fed diet HD varied from 2.5 to 6.6% as compared to 54.0 to 65.4% for the eggs of the hens fed diet HA. When diet HD was supplemented with 5% of rice bran, the average hatch of the fertile eggs was 14%. When diet HD was supplemented with an alcohol precipitate of the water extract of dried brewer's

yeast, a material found by Schumacher and Heuser ('39) to be a rich source of the new factor and reasonably free of the antidermatosis vitamin and riboflavin, the average hatch of the fertile eggs was 8.7%. The slight increase in reproduction obtained by adding either one of these supplements to diet HD is not believed to be significant. On the other hand, when diet HD was supplemented with both rice bran filtrate and the alcohol precipitate, the average hatch of the fertile eggs was 50.6%, a striking increase in reproduction above that obtained on diet HD alone or when supplemented with either one of these materials.

When the second experiment was in progress, Jukes ('39) and Woolley, Waisman and Elvehjem ('39) presented evidence strongly indicating that the antidermatosis vitamin is identical with pantothenic acid, obtained from liver by Williams, Truesdail, Weinstock, Rohrmann, Lyman and McBurney ('38). In this study the filtrate obtained by treating a water extract of rice bran with fuller's earth prevented the development of dermatosis in chicks fed diet CD and markedly increased the hatch of the fertile eggs of hens fed diet HD. The few chicks that hatched from the eggs of hens fed diet HD developed dermatosis much sooner, when fed a diet deficient in the antidermatosis vitamin, than normal chicks. This is evidence that the eggs produced by hens fed diet HD contained less antidermatosis vitamin than normal eggs. While these results are suggestive, it is impossible to conclude from them that the factor in rice bran filtrate necessary for reproduction in hens fed diet HD is identical with the antidermatosis vitamin in the sense to which it now appears restricted by the discovery of Jukes, and of Woolley and associates.

SUMMARY

In experimental work on the role of the antidermatosis vitamin in the nutrition of the mature fowl, an experimental diet deficient in the vitamin was developed by subjecting the cereal portion and the liver extract to prolonged dry heat treatment and the casein to special purification. This diet was shown to

be adequate in vitamins A, B, B_4, B_6, D, E, G, K, and the anti-encephalomalacia factor. Nicotinic acid was found either to be present in the diet or else not needed in avian nutrition.

The hen diet modified to make it adaptable for feeding chicks was found, when supplemented with the antidermatosis vitamin, to be deficient in another factor required for chick growth, not identical with any of the known vitamins. The new factor was shown to be destroyed by prolonged dry heat treatment, stable in yeast to autoclaving, water-soluble, adsorbable upon fuller's earth and present in milk, liver, yeast and fresh green grass.

Both the antidermatosis vitamin and the new factor were found to be necessary for reproduction in the domestic fowl. Supplementing the deficient diet with both these factors, however, failed to promote normal reproduction or to maintain the weight of the hens, but egg production was satisfactory. Whether these results were evidence that an insufficient quantity of the new factor was fed or that the experimental diet was deficient in another factor, or factors, required for reproduction and maintenance of weight, could not be determined from the evidence.

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A QUANTITATIVE CHEMICAL STUDY OF THE URINARY EXCRETION OF THIAMINE BY NORMAL INDIVIDUALS [1]

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SEVEN FIGURES

The presence of thiamine in human urine was first demonstrated by Muckenfuss ('18) and this finding subsequently confirmed by van der Walle ('22). Recent studies have been concerned with the quantitative estimation of the vitamin in urine using both biological (Harris and Leong, '36; Roscoe, '36; Knott, '36; Helmer, '37; Leong, '37; Light, Schultz, Atkin and Cracas, '38) and chemical (Westenbrink and Goudsmit, '37 and '38 a; Karrer, '37; Ritsert, '38) methods of assay. For the latter purpose the thiochrome procedure has been used exclusively.

In the present study the thiamine content of urines from normal adults was determined by the method of Melnick and Field based upon the reaction between the vitamin and diazotized p-aminoacetophenone and described in detail elsewhere ('39 b). It has been shown to be specific. No attempt has been made in the present study to insure the excretion of an acid urine because the work of Melnick and Feld just cited showed this to be unnecessary. Since these authors have also shown

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[2] Upjohn Fellow in Clinical Research, 1937–1940.

[3] Upjohn Fellow in Clinical Research, 1938–1940.

that thiamine in urine is in the free nonphosphorylated form, only estimations of the free compound were made.

The amount of thiamine in the feces was not determined in the present study. Such a determination is of questionable value since the normal fecal thiamine content appears to be constant and independent of the dietary intake, and arises practically entirely from bacterial synthesis in the lower part of the intestine (Leong, '37). This synthesized vitamin is retained in the bacterial cells and excreted with the feces (Abdel-Salaam and Leong, '38). Furthermore, no appreciable amounts of thiamine are excreted into the gut, even when large amounts of the vitamin are present in the tissues (Leong, '37).

Investigations of the factors influencing the urinary excretion of thiamine were necessary before significance could be attached to the values obtained.

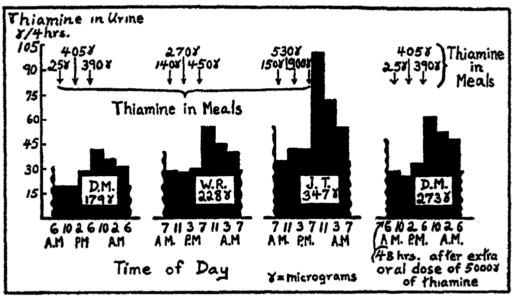
Diurnal variation in the urinary excretion of thiamine and its significance

Westenbrink and Goudsmit ('37) have reported a diurnal variation in the urinary excretion of thiamine and suggested that this may be due to a partial thiamine deficiency in the morning. Our findings indicate that the normal diurnal variation is due to an appreciable lag in excretion of the vitamin ingested in the diet and that the low values during the morning are not associated with any latent deficiency.

The urines from each of three normal male subjects, subsisting on adequate diets of calculated thiamine content,[4] were

[4] In the present report calculations of the thiamine contents of all the diets were based upon the tables compiled by Williams and Spies ('38). It is preferable to use their values in conducting balance studies with thiamine since the authors have attempted to estimate the true vitamin content with correction made for the amount of fat present. The vitamin B_1-sparing action of fat has been shown to be due to the decreased metabolic requirements for thiamine when the fat content of the diet is increased (Stirn, Arnold and Elvehjem, '39) and not to the actual presence of the vitamin in the foodstuff (Melnick and Field, '39 a). Thus, variations in ratio of fat to the non-fat constituents of each food can alter markedly the apparent thiamine content as estimated by methods of biological assay.

collected during six consecutive 4-hour periods and analyzed separately. The results obtained are presented in figure 1. The test was repeated with one of the subjects, subsisting on the same diet, but this time 48 hours subsequent to the ingestion of an extra oral dose of 5 mg. of thiamine (fig. 1). It is improbable that any latent thiamine deficiency existed since the subject now excreted an amount of thiamine, 273 micrograms, far in excess of those recorded for this individual while ingesting the constant diet (table 2). The values for each 4-hour period were now approximately 50% greater than before



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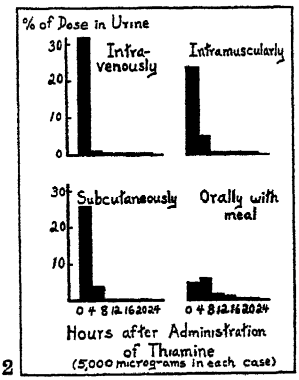

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Fig. 1 Normal diurnal variation in the urinary thiamine values.

Fig. 2 Urinary excretion of thiamine when administered parenterally and orally to the same normal subject, corrected for the basal excretion of thiamine.

with the previously observed relationship in thiamine excretion during the successive periods remaining unchanged. Also the diurnal variation observed in one subject while on a normal diet disappeared when he subsisted on a thiamine-free diet of washed polished rice, butter and dextrose.

Urinary excretion of thiamine following the administration of a test dose of extra vitamin

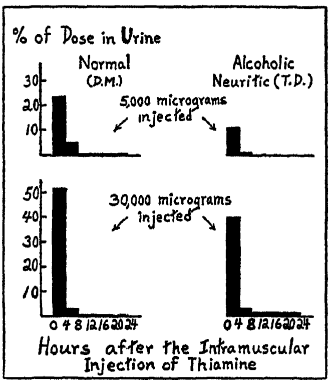
A 5 mg. test dose of the vitamin was administered both parenterally and orally to the same three subjects used in the study of the diurnal variations in thiamine excretion. In these

tests the individuals subsisted on the same diets as previously used so that corrections could be made for the amount of the vitamin normally excreted in the corresponding periods (fig. 1). The percentages of the dose recovered in the urine, partitioned and collected from one of the subjects (D.M.) over the 24-hour periods are presented in figure 2. The results with the other subjects were essentially the same. When the vitamin is given parenterally most of the total amount excreted is found in the first 4-hour sample. The greatest amount recovered followed the intravenous injection of the vitamin. A more gradual and a smaller total excretion is observed when thiamine is taken orally with a meal.

In spite of the advantage of the parenteral route in eliminating variations in rate and degree of intestinal absorption, we agree with Westenbrink and Goudsmit ('38 b) that the oral route is preferable for the administration of a single test dose. The objection to the use of the parenteral routes arises from the fact that the organism is rapidly 'flooded' with the vitamin with the major excretion after the test dose occurring within the first 4-hour period. Figure 3 illustrates that such a flooding effect occurs in avitaminotic individuals and tends to mask the difference in excretion between such persons and those normally nourished. The alcoholic neuritic excreted approximately 30% as much thiamine as the normal individual when no extra vitamin was given. When the test dose was injected, large amounts of thiamine were recovered from the urine. When the dose was increased, the percentage recovery of the extra thiamine from the urine of this patient showed relatively a much greater gain (300%) as compared with the normal (100%). The difference in thiamine excretion between these individuals was now largely eliminated. Clinical use of the oral test dose for supplementing information obtained by analyzing the usual 24-hour urine specimens has proved very valuable (Robinson, Melnick and Field, '40).

Further studies, presented in figure 4, indicate the importance of giving the oral test dose with a meal. When an aqueous

solution[5] of 5 mg. of thiamine is taken 12 hours after the last meal and 3 hours prior to breakfast, the fraction excreted in the urine is much less than when the same dose is taken with the largest meal of the day.[6] Apparently a considerable part of the vitamin taken into the fasting stomach, is destroyed prior to absorption. However, the major excretions of thiamine during the first 4-hour periods suggest that the vitamin



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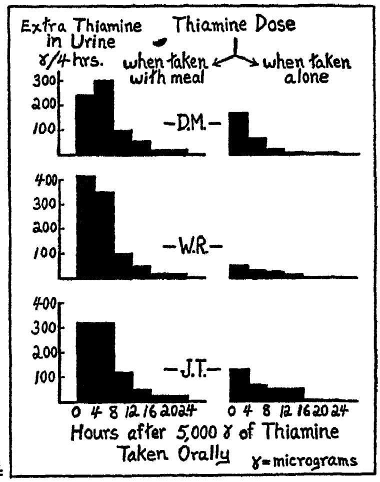

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Fig. 3 'Flooding' effect in both normal and avitaminotic subjects when the test dose of extra thiamine is administered parenterally. Correction was made for the basal excretion of thiamine.

Fig. 4 Difference in the urinary excretion of thiamine when the oral test dose of extra vitamin is taken with a meal and without a meal with the normal subject now post-absorptive. Correction was made for the basal excretion of thiamine.

is absorbed more rapidly under these conditions. Investigations of the stability of thiamine in the gastro-intestinal secretions under conditions simulating those in vivo are now in progress.

[5] In such a solution, adjusted to a pH acid to congo red and stored in the refrigerator, there will be no destruction of the vitamin for a period of at least 1 month.

[6] In these studies the subjects subsisted on the same diets used in the study of the diurnal variations (fig. 1) so that corrections could be made for the amount of the vitamin normally excreted.

The 24-hour urinary excretion of thiamine and the response to an oral test dose of extra vitamin

Fifteen normal men and ten normal women, from 25 to 35 years of age, were used in this study. The subjects were hospital staff members and their wives, subsisting on supposedly adequate diets composed of a wide variety of foods. Selection of foods during the test period was left to the choice of the subject and represented what that individual generally ate. Each subject ate the same diet for at least 2 consecutive days and two corresponding 24-hour urine specimens were collected.[7] Just prior to the collection of the second sample and immediately after the largest meal of the day an aqueous solution of 5 mg. of thiamine was taken orally. The first urine sample was analyzed to obtain the 24-hour urinary thiamine value when the diet furnished the entire supply of the vitamin. The second sample was analyzed to determine the total thiamine excretion after the oral dose was taken and, by subtracting from this the value for the preceding day, the per cent of the oral dose excreted in 24 hours was determined. The results of this study are presented in table 1.

The men were observed to excrete from 90 to 350 micrograms of thiamine per day, with the average value of 198. The latter represents an excretion of about 20% of the dietary intake. From 8 to 19% of the oral test dose was recovered from the urine, averaging 14%. The women excreted much less thiamine than the men, the values ranging from 61 to 146 micrograms of thiamine per day with an average value of 93 micrograms, or about 14% of the dietary intake. About 12% of the oral test dose was recovered from the urine on the following day.

Variations among the 24-hour urinary thiamine values obtained with these subjects cannot be attributed to differences in metabolism or in either the vitamin to calorie (Cowgill, '34) or in the thiamine to non-fat calorie ratio of the diet (Wil-

[7] The two 24-hour periods, during which the urine samples were collected, began in each case after dinner.

TABLE 1

Urinary excretion of thiamine by normal men and women before and after the oral administration of 5000 micrograms (γ) of extra vitamin

SUBJECT	WEIGHT (HEIGHT)	DIETARY THIAMINE INTAKE [1]	THIAMINE NON-FAT CALORIE RATIO	24-HOUR URINE VOLUME		THIAMINE EXCRETED IN URINE		FRACTION EXCRETED OF	
				Before oral dose	After oral dose	Before oral dose	After oral dose	Dietary intake	Oral dose [2]
males	*kg.* (*cm.*)	*γ/24 hrs.*		*cc.*	*cc.*	*γ/24 hrs.*	*γ/24 hrs.*	%	%
D.M.	74 (170)	820	0.5	900	1110	168	915	21	15
W.R.	68 (178)	860	0.5	1470	1100	228	1182	27	19
J.T.	66 (163)	1580	1.2	900	950	324	1236	21	18
D.L.	82 (183)	610	0.6	1830	1920	90	732	15	13
H.H.	77 (183)	1000	0.7	1740	2040	248	1025	25	16
H.F.	78 (173)	1130	0.9	2230	1310	237	740	21	10
C.K.	65 (175)	1130	0.8	1300	1940	171	600	15	9
H.A.H.	72 (173)	930	0.8	920	1080	146	550	16	8
K.H.	73 (183)	890	1.2	1220	1580	168	1120	19	19
J.S.	70 (170)	610	0.7	1400	1220	102	847	17	15
J.A.C.	68 (173)	?	?	1020	....	123	....	?	..
B.B.	71 (180)	?	?	1210	....	140	....	?	..
W.T.	59 (173)	?	?	730	....	350	....	?	..
R.C.	79 (183)	?	?	1510	....	201	....	?	..
J.C.	74 (190)	?	?	1730	....	269	....	?	..
Average male	72 (177)	960	0.8	1340	1430	198	895	20	14
females									
H.T.	50 (152)	750	1.0	1500	1110	95	606	13	10
F.S.	50 (157)	640	0.9	850	1200	68	500	11	9
E.L.	54 (157)	840	1.1	1030	1060	74	468	9	8
M.C.	57 (168)	840	0.8	2000	1800	80	774	10	14
K.S.	52 (152)	690	1.1	1910	1600	61	1034	9	19
D.F.	65 (163)	580	0.8	1460	800	77	800	13	14
A.H.	40 (152)	870	1.3	1080	730	146	720	17	12
E.S.	64 (168)	450	0.4	1550	1200	139	637	31	10
H.W.	47 (153)	?	?	780	810	100	726	?	13
B.L.[3]	(59) (165)	(340)	(0.5)	(1000)	(1020)	(40)	(366)	(12)	(7)
Average female	53 (158)	710	0.9	1350	1150	93	696	14	12

[1] Calculations of the thiamine contents of the diets were based upon the tables compiled by Williams and Spies ('38).

[2] Calculated by subtracting from the total thiamine, excreted after the administration of the oral test dose, the 24-hour urinary thiamine value of the preceding period and dividing the difference by 5000 micrograms.

[3] The values for this individual with respect to thiamine intake and excretion both before and after the administration of the oral test dose are so below the others that they have been eliminated in calculating the average values. This may be a case of latent deficiency.

liams and Spies, '38). The urine volume also appears to be a factor of insignificant importance in governing the urinary excretion of thiamine. Prolonged diuresis has been reported by Cowgill and associates ('30) to cause an earlier onset of vitamin B_1 deficiency in dogs subsisting on a diet inadequate in thiamine content. However, in the present study a single marked diuresis by one of the subjects, subsisting on a standardized diet but in addition forcing fluids, was not associated with any increase in the urinary thiamine values.

Further studies were carried out to establish the significance of the urinary thiamine values. Thirty 24-hour urine samples, collected from two of the male subjects over a period of 8 months, were analyzed. No attempt was made to eat the same diet during this period. The values obtained with one individual (D.M.) varied from 120 to 200 micrograms and with the other (W.R.) from 120 to 260 micrograms of thiamine. Some correlation was observed between thiamine excretion and the dietary intake. This marked variation in thiamine excretion by the same individual, however, is no longer apparent when the subject returns to the same diet for each test. This is shown by the representative data obtained with each subject and presented in table 2. Because of this reproducibility we feel that the values reported in table 1, although varying rather widely among the different individuals, are valid and do not represent any appreciable 'carry-over' effects of the previous diets. The response to the oral administration of extra vitamin is also reproducible. Large differences in the meals consumed by the subjects do not alter appreciably the characteristic response of the individuals to the oral test dose ingested along with these meals. The evening meals of two of the subjects (H.T. and J.T.), who ingested in these earlier experiments the same foods but different quantities of them, were now reversed and then followed immediately with the same oral test dose. The percentage excretions of the extra vitamin in each case were practically the same as when these subjects received the dose along with their regular meals.

In evaluating the adequacy of a patient's dietary régime, individual variation in thiamine excretion is no complicating factor. It is the minimal normal value which is of significance, 90 micrograms per 24 hours in the case of the males and 60 in the case of the female subjects (average-sized, sedentary adults). With patients giving reliable dietary histories of inadequate thiamine intake, the 24-hour urinary thiamine figures are always below these minimal normal values (Robinson, Melnick and Field, '40).

TABLE 2

Reproducibility of the values for the 24-hour urinary excretion of thiamine before and after the oral administration of extra vitamin

SUBJECT (SEX)	DATE [1] 1939	THIAMINE INTAKE		THIAMINE EXCRETION	
		Diet [2]	Oral dose	Before oral dose	After oral dose
		micrograms	*micrograms*	*micrograms*	*micrograms*
D.M.	1/30	820	5000	168	915
(♂)	3/27	820	5000	200	1002
W.R.	2/20	860	5000	198	1182
(♂)	3/20	860	5000	180	1200
J.T.	2/21	1580	5000	324	1236
(♂)	3/ 7	1580	5000	347	1212
H.T.	2/21	750	5000	95	606
(♀)	3/16	750	5000	89	561
Maximal deviation of results from the averaged values				9%	5%
Average deviation				5%	3%

[1] On the first day of the test, indicated by the recorded date, the diet alone furnished the sole source of thiamine. On the following day the 5 mg. dose of extra vitamin was taken with the subject still ingesting the same diet.

[2] Calculations of the thiamine contents of the diets were based upon the tables compiled by Williams and Spies ('38).

The response of the normal individual to inadequate thiamine intake

The diet used in this study consisted of washed polished rice, white bread, Cheddar cheese, butter, 20% cream, and dextrose, supplemented with adequate quantities of vitamin A, ascorbic acid, vitamin D and nicotinic acid. In table 3 the composition of this diet is compared with that of the normal basal diets fed these individuals. Attempts were made to keep con-

stant all variables but thiamine intake. One of the subjects (W.R.) subsisted on the experimental diet for a period of 32 days. After the twenty-second day the ration was supplemented with thiamine to give a total intake equal to that consumed in the normal basal diet. The vitamin supplement in aqueous solution was partitioned so that the total thiamine intake at each meal was exactly the same as when ingesting the normal diet. The results of this study are presented in figure 5. It will be observed that the urinary thiamine values decreased precipitously from the normal to values characteristic of avitaminotic individuals (Robinson, Melnick and Field,

TABLE 3

Comparison of the normal basal diets with the thiamine inadequate ration used in the present study

SUBJECT	DIET	THIAMINE INTAKE [1]	CALORIC INTAKE		THIAMINE NON-FAT CALORIE RATIO
			Total	Non-fat	
		micrograms	*calories*	*calories*	
W.R.	Normal basal	860	2900	1680	0.5
	Thiamine inadequate	260	2800	1670	0.16
	Inadequate + added thiamine	860	2800	1670	0.5
D.M.	Normal basal	820	2740	1700	0.5
	Thiamine inadequate	260	2800	1670	0.16

[1] Calculations of the thiamine contents of the diets were based upon the tables compiled by Williams and Spies ('38).

'40). At the time when vitamin supplementation was begun on the twenty-third day, the body stores of thiamine were obviously significantly reduced. Actually there was apparent definite clinical evidence of a deficiency characterized by aching calf muscles after progressively decreasing amounts of exercise, tenderness of calf muscles and Achilles tendons, and paresthesias of lower extremities. The low values of thiamine excretion persisted despite supplementation of the diet with the vitamin. In order to show that there were no unknown factors associated with the ingestion of the inadequate diet tending to augment the thiamine requirements, the subject

returned to his normal basal ration. The urinary thiamine values remained in the subnormal range. The per cent of the oral dose excreted in the urine at the end of the study, however, was comparable to the normal value characteristic for this individual. Apparently the thiamine stores of this individual were gradually replenished when the vitamin intake was returned to the normal control level, so that the excessive amounts normally available for excretion were not present.

In the studies by Harris and Leong ('36) the normal experimental subjects subsisted on the inadequate thiamine diet for

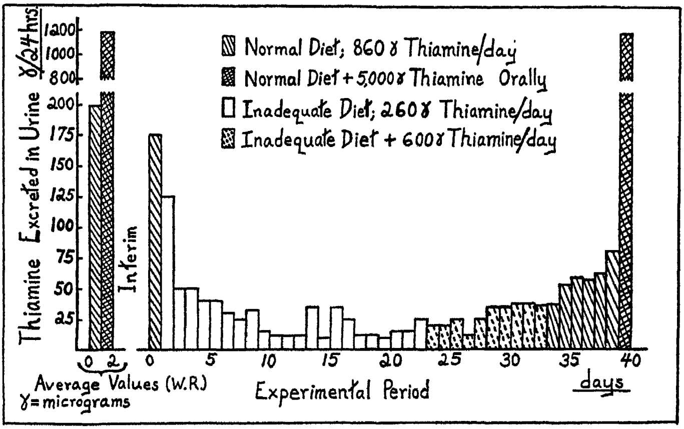
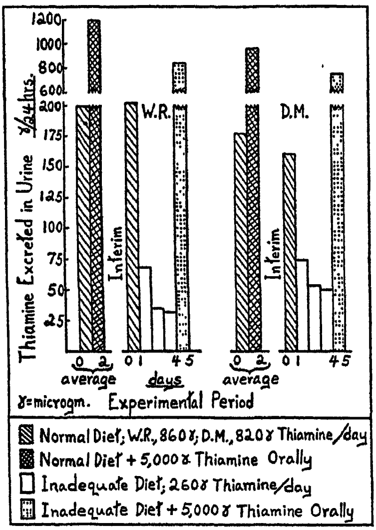


Fig. 5 Urinary excretion of thiamine by the normal individual subsisting on an inadequate thiamine diet and then on the same ration with added vitamin.

a period of only 3 days when the adequate diets were again ingested. They also observed the rapid decrease in the quantities of the vitamin excreted. However, the return to the normal values on re-alimentation was, in their studies, equally as rapid. Their findings would seem to indicate that the 24-hour urinary thiamine values reflect nothing more than the adequacy of the diet being consumed at the time of the test. However, from the results in the present study, the 24-hour values become of diagnostic importance in demonstrating objectively whether or not a true thiamine deficiency exists. A low value does not indicate irrevocably that the individual is

deficient. It may suggest simply that an inadequate diet was ingested shortly prior to and during the test. However, if the patient is given a normal diet a few days prior and during the day of the test and still excretes very small amounts of the vitamin, a deficiency may be considered to be present.

The use of an oral test dose, by minimizing the differences in available thiamine due to variations in dietary intake,



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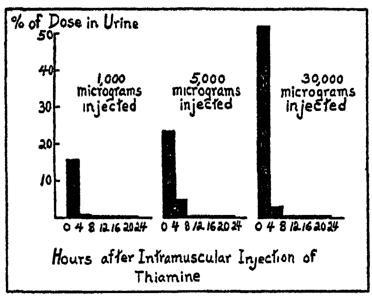

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Fig. 6 Normal urinary excretion of extra thiamine by normal subjects, subsequent to the ingestion of the oral test dose, despite the very low 24-hour urinary thiamine value preceding the test.

Fig. 7 Increase in the percentage excretion of thiamine in the urine as the amount available to the normal organism is increased. Correction was made for the basal excretion of thiamine.

should be of value in determining the degree of saturation of the body with the vitamin regardless of the adequacy of the diet being ingested at the time. When such a test is carried out, there should be available a sufficiently large excess of thiamine so that the normal organism ought to excrete a normal fraction of the test dose even though the previous 24-hour value may be low due simply to the inadequacy of the diet.

That this does take place is indicated from the studies presented in figure 6. In each case the percent of the oral dose excreted, correction having been made for the urinary thiamine value of the previous 24-hour period, was comparable to that found when the normal subject ingested an adequate diet.

It should be noted that the percentage of the ingested vitamin excreted in the urine decreases markedly when the normal intake is reduced to an inadequate level. In the study, summarized in figure 5, these values dropped from 20 to 5% of the dietary intake. That the percentage of the available vitamin, which is excreted in the urine, is a function of how great an excess is present is indicated in figure 7. In this study correction was made for the amount of thiamine excreted when the subject (D.M.) subsisted on the same normal basal diet with no extra vitamin administered (fig. 1). This response of the body to variable amounts of available thiamine may very well be the factor primarily responsible for the smaller percentage excretion of ingested vitamin in the case of the normal women subjects (table 1).

Return of the urinary thiamine values to the basal level subsequent to the daily oral administration of large quantities of extra vitamin

The procedures outlined in this report may be used to evaluate the results of thiamine therapy. Analysis of the urine at the time when a patient is receiving such therapy is worthless because flooding the organism with the vitamin, even in cases of deficiency, results in the excretion of a large amount of thiamine (fig. 3).

Two standardized subjects (D.M. and H.T., table 2) ingested daily 10 mg. of thiamine along with a normal adequate diet for a period of 23 days. This régime is comparable to the therapeutic measures to which thiamine-deficient patients are subjected. Subsequent to this period the subjects returned to the same basal diet used in the standardization of these individuals. Fully 2 weeks were required before the 24-hour uri-

nary thiamine values returned to the previous basal level. Accordingly, such a period of time should be allowed to elapse subsequent to therapy before conducting analyses of urines from treated patients. This period of 2 weeks is probably well above the minimal limit, since this study was conducted with normal subjects. Deficient individuals have in addition another pathway for the removal of the large amounts of thiamine administered, namely, the replenishment of the thiamine stores in the tissues.

Application of the observations made in the present study to problems of clinical interest

As a result of our studies of the urinary excretion of thiamine by normal individuals, several fundamental observations have been made which lend themselves to clinical application. The standards, which we have set up, are presented in table 4 and should apply to studies using procedures similar to our own.

Studies of clinical cases of thiamine deficiency (Robinson, Melnick and Field, '40) support the validity of the interpretations which have been applied to the urinary thiamine values. In our investigations of normal and clinical cases more than 120 subjects were used. In about 80% of these a close parallelism was observed between the 24-hour urinary thiamine values and the fractions of the oral test dose excreted. In the remaining 20%, with practically no exception, the responses to the administration of the oral dose were normal despite the very low 24-hour urinary thiamine values preceding the tests. This disagreement has been interpreted as indicating an inadequacy in the diet ingested immediately prior or during the test but as yet associated with no concomitant depletion of the thiamine stores of the organism. Low urinary thiamine values, subsequent to the administration of the oral test dose to patients with normal 24-hour values for the period prior to the test, were practically never observed.

TABLE 4

Standards for the evaluation of the nutritional status of adult subjects with respect to thiamine

ADULT MALE SUBJECTS		INTERPRETATIONS	ADULT FEMALE SUBJECTS	
24-hour urinary thiamine value	Fraction of oral dose excreted in 24 hours[1]		Fraction of oral dose excreted in 24 hours[1]	24-hour urinary thiamine value
micrograms	%		%	*micrograms*
above 100	7–20	Normal subject subsisting on a normal diet.	7–20	above 70
	1– 7	Subject deficient but receiving thiamine therapy during the time of the test.[2]	1– 7	
90–100	10–20	Subject normal; diet consumed during test is inadequate or probably borderline with respect to thiamine content.	10–20	60–70
	7–10	Subject borderline with respect to thiamine stores; diet characteristically eaten by subject.	7–10	
	1– 7	Subject approaching a deficiency; diet of very high thiamine content ingested during test.[2]	1– 7	
60–90	10–20	Subject normal; diet consumed during test is inadequate with respect to thiamine content.[3]	10–20	40–60
	7–10	Subject mildly deficient; diet may or may not have been adequate in thiamine content during day of test.	7–10	
	1– 7	Subject deficient, diet probably adequate during day of test.	1– 7	
0–60	10–20	Subject normal; diet consumed during test is very inadequate with respect to thiamine content.[3]	10–20	0–40
	7–10	Subject mildly deficient; diet may or may not have been adequate in thiamine content during day of test.	7–10	
	1– 7	Subject markedly deficient; diet may or may not have been adequate.	1– 7	

[1] Correction is made for the thiamine excreted during the preceding 24-hour period when the subject consumed the same ration but with no test dose of extra vitamin administered.

[2] It is conceivable that an individual may have an abnormally low capacity for the absorption of the vitamin, but because of the daily ingestion of a diet very rich in thiamine no deficiency occurs. Under such circumstances it then becomes possible that the 24-hour urinary thiamine value may be well within the normal range but the percentage recovery of the oral dose may be low. However, in our studies we have practically never observed such a relationship in thiamine excretion before and after the ingestion of the oral dose unless the tests were conducted with an individual deficient in thiamine and receiving therapy.

[3] It is likewise conceivable that an individual may be deficient but, because of an inability to utilize the vitamin though available, a large fraction is excreted in the urine. For example, the phosphorylation of thiamine may be assumed to occur principally in the liver (Ochoa and Peters, '38) and the vitamin has been shown to exist, in the organism for the most part in the phosphorylated state (Westenbrink and Goudsmit, '38 b; Ochoa and Peters, '38) as a coenzyme in one of the important enzyme systems in the body (Lohmann and Schuster, '37). An injury to the phosphorylating mechanism may result in the inability of the body to use the vitamin. However, the possibility of such an alteration in thiamine metabolism has never been demonstrated. Furthermore, our studies of cases of thiamine deficiency with very marked liver disease, who received thiamine intravenously over a period of several hours, have indicated that the vitamin is retained to the same extent as in patients with uncomplicated avitaminoses (Field, Robinson and Melnick, '40).

SUMMARY

The reaction between thiamine and diazotized p-aminoacetophenone in alkaline solution has been used for the chemical determination of the vitamin in urine. There is a diurnal variation in thiamine excretion due to an appreciable lag in the excretion of the vitamin subsequent to its ingestion as part of the meals. The responses of the standardized subject to both the oral and parenteral administration of a test dose of extra thiamine have indicated that the former procedure is more desirable for evaluating the nutritional status of the individual with respect to this vitamin. The average 24-hour urinary thiamine values of fifteen normal men and nine normal women were 198 and 93 micrograms respectively; 14% of the oral test dose of 5 mg. of extra vitamin were excreted by the men during the first 24-hour period following ingestion and 12% by the women. Variations in these values among the normal subjects were not related to differences in urine volume, in metabolism or in either the vitamin to calorie or thiamine to non-fat calorie ratio of the diet. Urinary thiamine values of normal individuals ingesting a constant diet are reproducible. During the production of an experimental thiamine deficiency the 24-hour urinary values drop precipitously to the avitaminotic range but rise slowly during recovery. The response of the subject to the oral test dose of extra thiamine is governed by the nutritional status of the individual and is independent of the adequacy of the diet consumed at the time of the test. The percentage of the available vitamin, which is excreted in the urine, is a function of how great an excess is present. Subsequent to flooding the organism with thiamine a period of 2 weeks is required to elapse before the urinary values return to the normal basal level. Standards are given for the interpretation of the urinary thiamine values in the diagnosis of thiamine deficiency.

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THE ISOLATION OF DESIRED QUALITIES OF RADIANT ENERGY FOR BIOLOGICAL EXPERIMENTATION [1]

LOYD A. JONES AND CLIFTON TUTTLE

SIX FIGURES

(Received for publication April 3, 1939)

Equipment for the isolation of a number of fairly narrow spectral regions of continuous radiation in the infra-red, visual and ultra-violet was described by one of us in a previous paper (Jones, '29). The source of radiation in this equipment was a carbon arc, and various filters were used beneath the arc to absorb unwanted regions. An area about 1 foot by 1½ feet was radiated to energy levels ranging from 0.003 calories/cm.^{2}/min. in the ultra-violet to 0.122 calories/cm.^{2}/min. in the infra-red.

The findings of Luce-Clausen who made a study of the biological action of these radiation bands on the rat (Luce-Clausen, '29) indicated that further experiments particularly designed to compare the near infra-red and visual regions might prove significant. The additional requirements of these proposed experiments as set forth by Luce-Clausen were two-fold: first, that considerably larger areas should be radiated to provide for simultaneous treatment of a larger number of animals to place the experiment upon a sound statistical basis; second, that the equipment should be capable of either continuous operation or at least of operation for a much longer time than was possible in the first series of experiments.

[1] Communication no. 720 from the Kodak Research Laboratories, Rochester, New York.

A table space of about 3 feet by 9 inches is necessary for the requisite number of animals to be exposed in each spectral region. It is desirable to maintain over this entire area an energy level, continuous in each band, of the order of 0.05 calories/cm.^{2}/min.

Choice of light source. Because a light source emitting a continuous spectrum was essential, gaseous discharge arcs were eliminated.[2] The frequent attention required for the operation of carbon arc lamps makes these impractical for the present purpose.

The only source available was the incandescent tungsten lamp. From the point of view of ease of maintenance, cleanliness and economy, the incandescent lamp is thoroughly satisfactory. As a radiator in the near infra-red it is also good, but as a source of a considerable amount of energy in the visual it leaves much to be desired, especially when this visual radiation must be freed from infra-red. Figure 1 shows the spectral energy distribution of a tungsten lamp at 3000° K. (1000-hour life rating). Only about 14% of the total energy emitted by this lamp lies in the visual and most of the remainder in the infra-red.

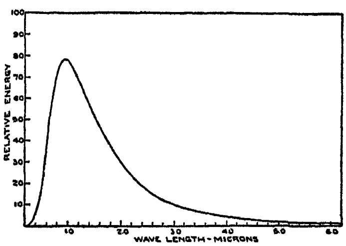
Choice of filter materials. In the previous experiment (Jones, '29) there was much flexibility in the choice of filter constituents because the area to be radiated was small and the time of radiation was short. The best absorbers of the long wavelengths are liquids. In the present experiment which had to be carried on continuously over larger areas, liquid filters were out of the question for obvious reasons. The problem of isolating the two spectral regions consists then in selecting the most efficient filter materials for the available solids, such as special glasses and dyed gelatins.

Isolation of the visual. Probably the best available material for the separation of the infra-red from visual radiation is Corning Aklo glass. The curve in figure 2 shows the optical

[2] The fluorescent tubes excited by gaseous discharge arcs which have recently become commercially available emit nearly continuous radiation in the visual spectrum and might have proved useful for this experiment had they been available at the time.

density of the medium grade of this glass plotted against the wavelength. It should be noted that there is a marked transmission band at 500 mμ where the density is 0.4 (transmittance about 40%) and that toward the longer wavelengths the density rises sharply to 3.0 (transmittance 0.1%) at 760 mμ. The density remains above 3.0 throughout the near infra-red region but decreases again for wavelengths longer than 1.6 μ.

With this glass alone covering the tungsten source, there would be an appreciable amount of long wavelength energy transmitted. An additional source of very long wavelength radiation is the Aklo glass itself which becomes heated by virtue of the energy which it has absorbed and may radiate strongly unless it is effectively cooled.



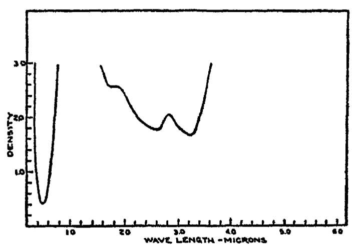


Fig. 1 Spectral energy distribution of a tungsten lamp at 3000° K.
Fig. 2 Optical density of Corning 'Medium' Aklo glass.

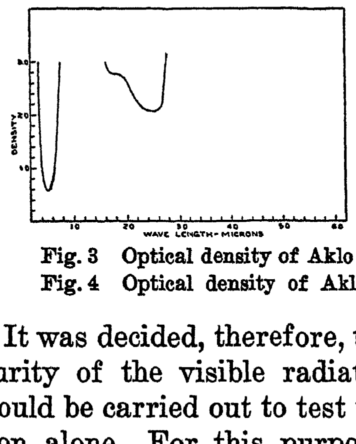
Ordinary double-thickness window glass is an effective shield from the far infra-red radiation. With two layers of such glass separated from each other and from the Aklo glass by air spaces, much of the unwanted long wavelength energy originating in or transmitted by the Aklo glass may be prevented from reaching the working plane.

The absorption characteristic of the combination filter (Aklo plus two layers of window glass) is shown in figure 3.

Isolation of far infra-red. Preliminary experiments showed that there would be great difficulty in eliminating from the visual radiation all of the extreme long wavelength infra-red. When the combination filter just described was exposed for any considerable length of time to radiation, convection and conduction of heat from the requisite number of high-wattage

tungsten sources, there was a rise in temperature of the lowest layer of window glass. Even a high velocity stream of air forced between the glass layers failed to prevent this temperature rise altogether. Had it been economically feasible to cool the lowest plate by a stream of water, the difficulty might have been overcome, but there seemed to be no effective and practical alternative.

This secondary radiation has a maximum in the neighborhood of 10 μ, far beyond the regions with which this experiment was designed to deal. Nevertheless, there seems to be no justification for assuming a priori that this radiation has no biological effect.



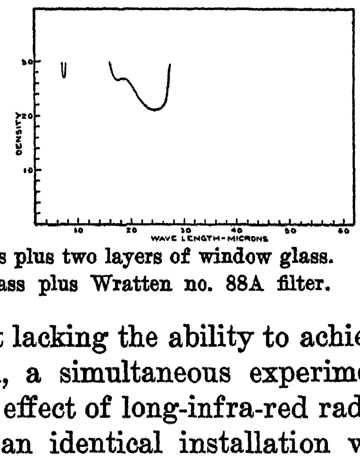


Fig. 3 Optical density of Aklo glass plus two layers of window glass.
Fig. 4 Optical density of Aklo glass plus Wratten no. 88A filter.

It was decided, therefore, that lacking the ability to achieve purity of the visible radiation, a simultaneous experiment would be carried out to test the effect of long-infra-red radiation alone. For this purpose an identical installation was made, using Aklo glass combined with a filter (Wratten no. 88A coated on glass) which completely absorbed the visible region transmitted by the Aklo glass. The density-wavelength characteristic of this combination is drawn in figure 4. It will be seen that the ultra-violet, visible and near infra-red are completely eliminated. There is a small transmission band at approximately 2.4 μ but the maximum transmission here is less than 1%. By using a few tungsten lamps mounted over the compound filter, it is quite easy to maintain the energy level at a value appreciably above the amount of long wavelength impurity which is unavoidably present along with the visual.

Isolation of the near infra-red. The third quality of radiant energy that was desired for this experiment may be referred to as the near infra-red. This was isolated by using a Corning glass filter no. 585 in combination with the infra-red (Wratten 88A) filter previously referred to. The density wavelength characteristic for this combination is shown in figure 5, in which it is seen that there is a double transmission band lying between 0.75 and 2.8 μ. There is a small band at about 4.0 but here the transmission is less than 1%.

Arrangement of the equipment. Four different chambers separated from one another by partitions were prepared for the radiation experiment. The actual setup is made clear by

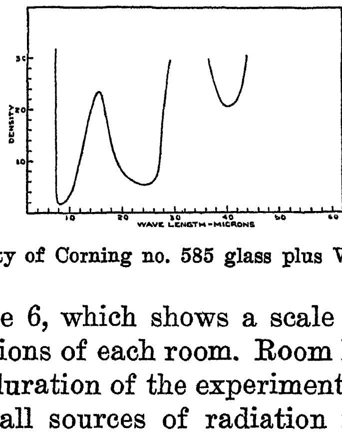


Fig. 5 Optical density of Corning no. 585 glass plus Wratten no. 88A filter.

reference to figure 6, which shows a scale plan of the whole and typical elevations of each room. Room I was kept in total darkness for the duration of the experiment and an effort was made to remove all sources of radiation from the working table where a control group of animals was kept. Rooms II, III and IV were the near infra-red, the visual and the far infra-red rooms, respectively. The arrangement in each was the same except for differences in filters and lamp wattages.

The rat cages, 10-inch cubicles of wire mesh, were arranged on tables immediately beneath the horizontal filters. The filters were framed in metal sash, as shown in the insert in the figure. Above the filters is the lamp chamber. The upper and lower filters formed the top and bottom of a duct through which air was pumped at a velocity of 700 feet per minute. Outside air was drawn into the duct through cleaning filters.

The air stream passed consecutively through the filters in each of the four rooms. A portion of the air was diverted through the lamp chamber to help remove heat dissipated by the lamps.

In room II, because of the fact that incandescent tungsten emits large amounts of energy in the region transmitted by the filter, it was only necessary to use twenty 300-watt lamps in order to obtain the desired level of near infra-red radiation. The filter materials do not absorb in regions where large quan-

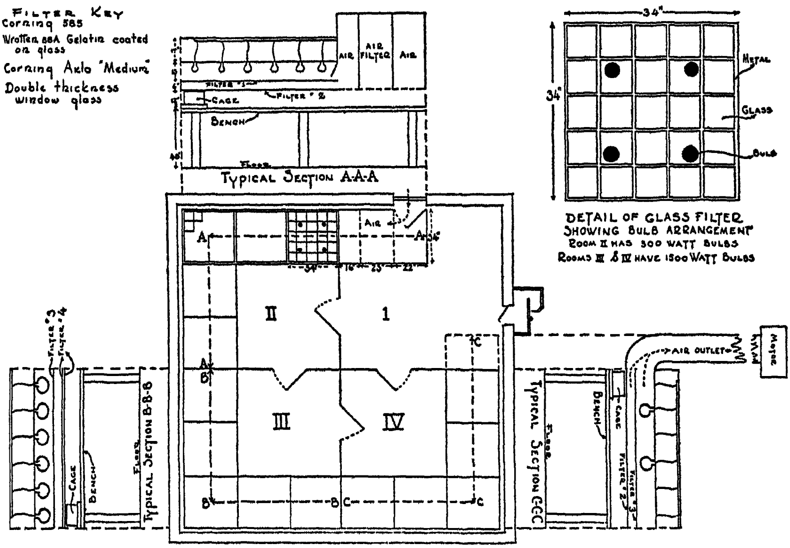


Fig. 6 Layout of radiation chambers

tities of energy are emitted and consequently they do not heat up appreciably. Hence, there is no problem of far infra-red impurity in room II.

In the visible radiation room III, twenty 1500-watt lamps were used and even with this amount of lighting the energy level was lower than was desired. Here it was that the heating of the filters gave rise to large amounts of far infra-red.

In room IV it was found that the air passing through the ducts of rooms II and III had become heated to such an extent that the filters were warmed almost to a sufficient tempera-

ture to radiate the desired amount of the far infra-red. This was particularly true during the summer when the incoming air was already warm. A few 1500-watt lamps were installed in the lamp chamber of room IV to make up the deficit in far infra-red which occurred during the winter.

An ambient temperature of 75° F. was maintained in all of the rooms throughout the course of the experiment by means of a York refrigeration unit. The air was conducted down into each room by metal pipes connected overhead with the fan of the refrigeration unit.

TABLE 1

Radiation in rooms II, III and IV in gm. cal./min./cm.²

ELAPSED TIME	ROOM NO. II G 585 + 88A		ROOM NO. III [1] Aklo + two window glass		ROOM NO. IV Aklo + 88A
	Near infra-red	Secondary	Visual	Secondary	Secondary
minutes					
Start	0.065	0.0	0.02	0.0	0.0
5	0.1	0.0	0.025	0.0	0.002
10	0.1	0.0	0.032	0.0	0.075
15	0.1	0.0	0.028	0.0	0.11
20	0.10	0.005	0.030	0.0	0.14
25	0.10	0.005	0.027	0.0	0.15
30	0.10	0.005	0.031	0.005	0.15
35	0.10	0.006	0.029	0.01	0.155
40	0.10	0.006	0.030	0.015	0.160
45	0.10	0.006	0.030	0.025	0.160
60	0.10	0.010	0.03	0.05	....
360	0.09	0.01	0.029	0.055	0.16

[1] The visually determined illumination in room III was approximately 300 foot candles.

Energy measurements and data. Seventeen-element Moll thermopiles were permanently installed in each of the rooms. These were calibrated with a standard Nernst burner. Thermoelectric output was measured with a Rawson 0 to 0.5 millivoltmeter. Connections were made through individual switches to a central reading station so that frequent checks on the operation of the equipment could be readily made.

A typical set of data is given in table 1.

Each thermopile of course reads total energy and does not discriminate between secondary and desired radiation. The secondary radiation increases steadily as the filters warm up, usually reaching a steady state after about an hour of operation. The determination of the amount of secondary present is made by interposing a sheet of the ordinary window glass over the thermopile and taking a reading before the glass has become warmed appreciably above room temperature. The readings in all the rooms are fairly consistent with the exception of the apparent increase in the visual which continues for the first 20 minutes. A possible explanation for this effect is the gradual removal of moisture on the filters by the warm stream of air. During the hottest days of summer there was some increase in secondary radiation in all rooms.

The equipment was turned on and off by a Sangamo time switch and operated for a 12-hour period each day from October, 1936 to April, 1938. During that time the only attention required was replacements of the lamps at 10-week intervals.

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THE DETERMINATION OF ASCORBIC ACID IN COMMERCIAL MILKS [1]

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Milk as a source of vitamin C has attracted considerable attention recently. Holmes, Tripp, Woelffer and Satterfield ('39) have reviewed available data and have studied the breed and seasonal variations in the ascorbic acid content of certified milk from Guernseys and Holsteins. Our interest lies in the ascorbic acid content of regular commercial samples of milk as related to the quantity present in the milk at the time of milking.

EXPERIMENTAL PART

The procedure described by Mindlin and Butler ('38) which involves the use of the Evelyn photoelectric colorimeter ('36) for the determination of ascorbic acid in blood plasma was modified so as to be applicable to milk.

The oxidation of ascorbic acid other than by 2, 6,-dichlorophenolindophenol even in the presence of added copper is prevented by the use of a solution developed by Willberg ('38) which consists of sodium chloride and oxalic acid. It was modified by us to contain also metaphosphoric acid with the result that a clearer serum is obtained and the formation of a precipitate in the colorimeter tube is prevented when the dye-acetate solution is added. Loss of reducing activity is prevented in the filtrate for 16 hours if exposure to direct sunlight or strong illumination is avoided.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The volume of milk serum and quantity of dye-acetate solution are so controlled that the usual concentration of vitamin present reacts almost instantaneously. Under these same conditions the interfering substances formed in the milk after hydrogen sulfide treatment react at a slower rate, thus enabling their interference to be largely eliminated by extrapolation to zero time.

Among the interfering substances which are formed upon treatment of the milk with hydrogen sulfide, no doubt one of them is reduced riboflavin. Experimentation with ascorbic acid-riboflavin solutions under similar conditions indicates this to be the case. Apparently more riboflavin is reduced, the longer the milk serum remains in contact with the reductant, 2 hours being the average time when the effect first becomes noticeable. Fortunately, the reduced riboflavin and other substances react more slowly than ascorbic acid under the conditions used and therefore this interference is largely eliminated by extrapolation. It is to be noted that a titrimetric procedure employed on a milk serum which has been treated with hydrogen sulfide long enough to reduce the dehydroascorbic acid will invariably give results which are too high and the error involved will be dependent upon two factors—the time during which the serum has been left in contact with hydrogen sulfide and the time actually employed for the completion of the titration. Hence the use of the colorimeter or some similar instrument is essential for the determination of the dehydroascorbic acid if the effect of other reductants formed under the conditions needed to reduce the dehydroascorbic acid is to be largely eliminated.

The apparatus is identical with that used by Mindlin and Butler ('38) as is the 2, 6,-dichlorophenolindophenol, sodium acetate and indophenol acetate solutions. The potassium oxalate, cyanide and metaphosphoric acid solutions are, however, replaced by a modified Willberg ('38) reagent which is prepared accurately in order to insure the proper pH in the colorimeter tube, by dissolving in 100 ml. of glass-distilled water 0.6 gm. $H_2C_2O_4 \cdot 2H_2O$, 4.8 gm. NaCl C.P. and 6.5 gm.

HPO_3 (reagent, grade, sticks). This reagent is stable for 1 week at 15 to 20°C. and remains at constant pH.

Determination of ascorbic acid. In the absence of strong light 25 ml. of milk are pipetted into a 125 ml. Erlenmeyer flask containing 75 ml. of modified Willberg reagent. The protein precipitate is removed by filtering through paper of quality similar to Whatman 42.

Five milliliters of the filtrate are measured into a colorimeter tube and 10 ml. of the indophenol-acetate solution are added. The contents are stirred and read immediately.

Since it is not always possible to obtain a filtrate that is crystal-clear, it is recommended that the suggestion of Bessey ('38) be used; that is, a small crystal of ascorbic acid is added after the original reading has been made whereupon the correction due to the prevalent turbidity is determined. Thus the true reading is equal to the original reading plus 100 minus the reading after the crystal of ascorbic acid has been added.

Determination of dehydroascorbic acid. After the addition of a few drops of dibutyl phthalate to prevent foaming, hydrogen sulfide is bubbled through the remainder of the filtrate for 5 minutes. The tightly stoppered flask is allowed to stand for 8 hours in the dark at room temperature. The hydrogen sulfide is removed by passing a vigorous stream of wet oxygen-free nitrogen through the filtrate for 30 to 45 minutes. In order to remove the elemental sulfur and the dibutyl phthalate, the contents of the flask are filtered through paper of quality similar to Whatman 42.

Five milliliters of the filtrate are measured into one of the colorimeter tubes. With a simultaneous start of a stop watch, 10 ml. of the dye-acetate solution are blown into the tube and the galvanometer readings at 15, 30, 45, 60, 90 and 120 seconds are recorded. After the one hundred and twentieth second a few crystals of ascorbic acid are added and all these readings are corrected in the manner already described for the slight amount of turbidity that invariably seems to be present. The logarithms of the difference between

100 minus the galvanometer readings plotted against time and extrapolated to zero time will give the most accurate galvanometer reading due to the ascorbic acid plus the dehydroascorbic acid.

We have found that under the conditions described K has a value of 0.166 $\pm$0.003 for different lots of indophenol (practical). It is to be noted that when determining this value it is essential to have the same final pH in the colorimeter tubes as that obtained when a sample of milk is substituted for the standard ascorbic acid solution. This may readily be accomplished if the standard ascorbic acid solution employed is made by diluting three parts of modified Willberg reagent with one part of water. Other details and calculations have already been described by Mindlin and Butler ('38) and Bessey ('38). Typical recovery experiments have demonstrated a per cent recovery from 94 to 98.

DISCUSSION

Knight, Dutcher and Guerrant ('39) have shown conclusively that there is no adsorption of ascorbic acid on the precipitated proteins and are of the opinion that as the milk leaves the udder of the cow, its ascorbic acid is all in the reduced form. We have confirmed the latter point and have noted that, if the milk is immediately pipetted into the modified Willberg reagent directly after the milking machine is detached from the udder of the cow, treatment with hydrogen sulfide is unnecessary as no significant oxidation occurred during the milking operation and that the reagent prevented any further oxidation until the analysis could be completed.

Results of determinations by method presented. Recorded in table 1 are the analyses of milks of Holstein, Brown Swiss, Jersey and Guernsey cows in the regular University of Wisconsin herd. These analyses were made in January when the animals were on winter feed. The samples were secured immediately after the machine was detached from the udder and pipetted directly into the Willberg reagent. The samples were collected on 3 consecutive days during the afternoon

TABLE 1

Daily variations in the ascorbic acid content of milk from individual cows taken directly after milking

HOLSTEIN (A)[1]	ASCORBIC ACID PER LITER			PM MILKING POUNDS MILK
	1st day	2nd day	3rd day	
	mg.	*mg.*	*mg.*	
3	14.6	15.3	14.7	20
7	21.2	21.3	21.5	14
9	26.2	25.0	25.7	15
10	18.5	....	18.9	9
11	25.6	23.3	29.2	24
12	17.0	18.4	16.7	58
18	23.2	18.6	18.3	18
29	21.3	21.7	19.9	6
Average	20.9	20.5	20.6	
HOLSTEIN (B)[1]				
52	18.8	19.5	19.1	5
58	28.6	28.4	23.6	7
63	23.9	23.0	23.6	10
64	17.6	18.6	18.6	8
65	21.7	23.0	21.2	14
67	23.0	25.1	25.1	21
83	22.1	17.1	19.1	8
84	22.0	21.3	22.4	8
91	19.9	20.0	20.5	17
Average	21.7	21.6	21.7	
BROWN SWISS				
805	23.0	22.0	23.6	12
811	28.6	26.6	28.6	23
815	20.0	18.6	19.1	19
814	27.5	27.5	27.4	11
822	23.6	23.3	23.2	7
827	26.4	26.8	25.6	6
834	28.0	29.3	28.6	8
839	25.8	26.2	25.1	11
840	....	20.0	29.2	9
Average	25.3	24.4	25.6	
JERSEY				
608	21.0	20.8	20.8	7
621	23.6	24.4	20.0	6
623	31.2	29.6	29.0	14
639	15.4	16.7	17.6	7
640	31.8	29.4	27.8	10
656	18.8	22.4	21.6	8
660	22.7	24.8	22.9	10
661	23.7	20.2	25.1	14
Average	23.5	23.5	23.1	
GUERNSEY				
404	22.5	21.6	22.0	8
412	23.3	22.0	23.6	20
417	22.7	22.7	24.2	13
421	21.7	24.0	23.3	17
424	19.2	19.6	20.0	13
427	27.5	29.6	29.6	9
428	24.4	23.9	25.2	11
432	24.4	24.2	24.8	10
437	18.6	16.8	17.7	9
438	21.7	22.7	23.3	13
441	22.5	24.8	24.0	13
447	20.5	22.1	21.0	7
450	18.8	20.4	21.2	10
455	18.0	18.0	18.0	15
457	20.8	20.0	22.7	16
415	26.6	27.2	27.5	11
Average	22.2	22.4	23.0	

[1] (A) group on winter feed the year round. (B) group had summer pasture available yearly.

milking. It will be noted that the daily values for the ascorbic acid are very constant for any individual animal, that the quantity of ascorbic acid produced per liter varies significantly with each animal and that a high producer does not necessarily give less ascorbic acid per liter than a low producer.

It is further observed that milk from the Brown Swiss herd contained the most ascorbic acid per liter followed by Jersey, Guernsey and Holstein, respectively, and that the magnitude of the figures for the Guernsey and Holsteins are in close agreement with those reported by Holmes, Tripp, Woelffer and Satterfield ('39).

Samples of raw, certified, certified Guernsey and certified vitamin D milks were collected at the different dairies throughout the city of Madison. These milks (table 2) on the average are only a little below the fresh milks as recorded in table 1, indicating that commercial raw and certified milks as delivered to the consumer lose only a small amount of their antiscorbutic potency. Likewise, samples of commercial pasteurized milks were collected and analyzed. On an average they contained only about one-half as much ascorbic acid as fresh raw milks and significantly less ascorbic acid than the commercial unpasteurized milks. These results are recorded in table 3.

In both the pasteurized and unpasteurized milks the amount of dehydroascorbic acid is less than the ascorbic acid. The average percentage of dehydroascorbic acid present in the pasteurized milks makes up less of the total than in the case of unpasteurized milks. This is probably indicative of the tendency of pasteurization to form more dehydroascorbic acid which, once formed, is more readily destroyed.

Commercial pasteurized vitamin D milks in which the potency has been increased by incorporating vitamin D concentrates directly into the milk by homogenization fall below the average value of ordinary pasteurized milks in respect to the ascorbic acid content (table 4). On the other hand, if the vitamin D content has been increased by feeding the animal irradiated yeast, the antiscorbutic property falls on a par with

TABLE 2

Ascorbic acid content of commercial raw milks

COMMERCIAL MILK	ASCORBIC ACID PER LITER		
	Reduced	Oxidized	Total
	mg	*mg*	*mg*
Grade A raw	7.9	8.8	16.7
Certified dairy 1	13.1	6.3	19.4
Certified dairy 2	13.7	4.8	18.5
Certified dairy 3	15.1	4.0	19.1
Certified dairy 4	12.8	8.8	21.6
Grade A Guernsey raw	7.1	13.0	20.1
Certified Guernsey dairy 1	8.2	5.0	13.2
Certified Guernsey dairy 2	16.2	2.5	18.7
Certified vitamin D from C.L.O. (1)	7.9	6.0	13.9
Certified vitamin D from C.L.O. (2)	7.9	4.2	12.1
Average	10.9	6.3	17.3

TABLE 3

Ascorbic acid content of commercial pasteurized milks

PASTEURIZED COMMERCIAL MILK	ASCORBIC ACID PER LITER		
	Reduced	Oxidized	Total
	mg.	*mg.*	*mg.*
Guernsey dairy 1	3.8	1.8	5.6
Guernsey dairy 2	14.0	2.6	16.6
Guernsey dairy 3	7.8	8.8	16.6
Milk dairy 1	5.7	3.8	9.5
Milk dairy 2	14.0	1.1	15.1
Milk dairy 3	10.1	3.4	13.4
Milk dairy 4	12.4	5.3	17.7
Milk dairy 5	6.2	3.2	9.4
Grade A dairy 1	1.9	5.5	7.4
Grade A dairy 2	14.0	1.1	15.1
Average	8.9	3.5	12.6

TABLE 4

Ascorbic acid content of commercial pasteurized and modified milks

PASTEURIZED COMMERCIAL MILK	ASCORBIC ACID PER LITER		
	Reduced	Oxidized	Total
Vitamin D grade A (irradiated ergosterol)	3.7	3.7	7.4
Vitamin D (irradiated ergosterol added)	4.1	5.7	9.8
Vitamin D (feeding irradiated yeast)	6.9	6.8	13.7
Homogenized vitamin D (feeding irradiated yeast)	1.7	9.4	11.1
Mineral modified	2.7	5.4	8.1

the average value for commercial pasteurized milks. This would indicate that homogenization tends to destroy ascorbic acid.

Mineral modified milk is low in ascorbic acid and this is doubtless to be expected considering the treatment to which the milk must be subjected.

SUMMARY

A convenient method for the determination of ascorbic and dehydroascorbic acid in raw and commercially pasteurized milks has been described. The photoelectric colorimeter makes possible the elimination of many of the interfering substances which are formed when the milk is treated with hydrogen sulfide. Among these substances is reduced riboflavin.

The influence of breed as well as milk production has been observed as affecting the quantity of ascorbic acid produced. No daily variation in the milk of individual cows was observed.

It was found that commercial raw milks contained an antiscorbutic potency which was only slightly less than fresh raw milks and that pasteurized milks on the average contained only one-half the latter potency. Mineral modification and homogenization apparently have a destructive effect on ascorbic acid.

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MEAD JOHNSON & COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1940 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute at New Orleans on March 13, 1940.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more parties. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1939 must be in the hands of the Secretary by January 5th, 1940. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

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SECRETARY, AMERICAN INSTITUTE OF NUTRITION

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